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Lynea Alene Murphy
University of Kentucky, lynealene@hotmail.com

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ABSTRACT OF DISSERTATION

Lynea Alene Murphy

The Graduate School
University of Kentucky

2008

IMPLICATIONS FOR THE HSF2/PRC1 INTERACTION AND REGULATION OF
CONDENSIN BY PHOSPHORYLATION DURING MITOSIS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy
in the College of Medicine
at the University of Kentucky

By
Lynea Alene Murphy

Lexington, Kentucky

Director: Dr. Kevin D.Sarge, Professor of Molecular & Cellular Biochemistry and
Graduate Center for Toxicology

Lexington, Kentucky

2008

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ABSTRACT OF DISSERTATION

IMPLICATIONS FOR THE HSF2/PRC1 INTERACTION AND REGULATION OF CONDENSIN BY PHOSPHORYLATION DURING MITOSIS

At the beginning of mitosis, chromosomes are condensed and segregated to facilitate correct alignment later in cytokinesis. Condensin is the pentameric enzyme responsible for this DNA compaction and is composed of two structural maintenance of chromosomes (SMC) subunits and three non-SMC subunits. Condensin mutations generate chromosomal abnormalities due to improper segregation, leading to genome instability and eventual malignant transformation of the cell. Cdc2 phosphorylation of the non-SMC subunits, CAP-G, CAP-D2, and CAP-H, has been demonstrated to be important for condensin supercoiling activity and function. While these subunits are thought to be phosphorylated by Cdc2, the exact sites have not yet been identified and characterized. The basis of this research was to determine the Cdc2 phosphorylation sites in the CAP-G subunit of the condensin enzyme and to characterize the functional significance of the sites in the regulation of condensin activity using site-directed mutagenesis and immunofluorescence microscopy.

While DNA condensation represents a critical step early in mitosis, formation of the mitotic spindle represents a vital event leading to the division of a cell into two daughter cells in a process known as cytokinesis. Protein regulating cytokinesis 1 (PRC1) is a mitotic protein essential for cytokinesis that participates in formation of the mitotic spindle in a phosphorylation dependent manner. PRC1 possesses microtubule bundling properties. Loss of PRC1 leads to mis-segregation of chromosomes and abnormal cytokinesis.

HSF2 is a transcription factor known to be important in development and differentiation. Previous research has determined that HSF2 plays a significant mechanistic role in the process of hsp70i gene bookmarking during mitosis. Bookmarking is an epigenetic phenomenon whereby certain gene promoters remain uncompacted, in contrast to the majority of genomic DNA during mitosis. This lack of compaction allows quick reassembly to a transcriptionally competent in G1 of the cell cycle and ensures the ability of the cell to induce expression of the cytoprotective hsp70i protein. HSF2 and PRC1 were found to interact in a yeast-two hybrid screen. Given the

importance of both of these proteins during mitosis, this study seeks to characterize the HSF2/PRC1 interaction and determine the potential role for PRC1 in hsp70i gene bookmarking.

KEYWORDS: PRC1, Condensin phosphorylation, cytokinesis, HSF2, bookmarking

Lynea Alene Murphy

August 6, 2008

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By

Lynea Alene Murphy

Kevin D. Sarge, Ph.D.
Director of Dissertation

David K. Orren, Ph.D.
Director of Graduate Studies

August 6, 2008
Date

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DISSERTATION

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University of Kentucky
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Chapter One

Review of Literature

Introduction

Condensin I

At the beginning of mitosis, DNA is condensed to facilitate correct segregation of chromosomes later during mitosis. Failure to properly condense and segregate chromosomes will eventually lead to aneuploidy which is characterized by abnormal chromosome number and is linked to birth defects and cancer (Legagneux *et al.*, 2004). The enzyme responsible for this critical step is condensin I, a pentameric enzyme originally identified in extracts of *Xenopus laevis* eggs (Hirano and Mitchison, 1994; Hirano *et al.*, 1997). Functional studies in *Xenopus* determined that condensin I introduces positive supercoils into DNA when topoisomerase I and ATP are present (Kimura *et al.*, 1998). Condensin I is composed of two core subunits, CAP-C (SMC4) and CAP-E (SMC2) which form a heterodimer and are members of an ATPase family known as structural maintenance of chromosomes or SMC (Hagstrom and Meyer, 2003). Characteristic of SMC proteins, there are two ATP molecules located at the distal ends of the heterodimer (Melby *et al.*, 1998). In addition to the two SMC subunits, the enzyme complex is composed of three unrelated non-SMC subunits, CAP-G, CAP-D2, and CAP-H (Hirano *et al.*, 1997; Kimura *et al.*, 1998) (Figure 1.1). A second class of condensin, known as condensin II shares the CAP-C and CAP-E subunits but differs in the non-SMC subunits (Ono *et al.*, 2003). Condensin I and II appear to differ in their roles in DNA condensation and also in their chromosomal localization as condensin II associates with chromosomes at prophase (Ono *et al.*, 2003; Ono *et al.*, 2004). It is unknown exactly how condensin II is regulated and what this enzyme contributes to the condensation process in mitosis.

In vivo studies of condensin I function

Condensin I binds to chromosomes early in mitosis after breakdown of the nuclear envelope in prometaphase (Ono *et al.*, 2004). Loss of condensin I leads to abnormalities in the kinetochore as well as the formation of chromosome bridges during anaphase (Ono *et al.*, 2004). The critical role for condensin I in chromosome architecture

and also segregation has been well studied across many different species since the condensin I complex is highly conserved from yeast to humans. Studies of the fission yeast *Schizosaccharomyces pombe* cut3 and cut14, which are homologues of SMC4 and SMC2 determined that temperature sensitive mutants of these proteins are unable to condense chromosomes (Saka *et al.*, 1994). All five subunits of fission yeast condensin I are homologous to *Xenopus laevis* condensin I and all are necessary for cell viability (Sutani *et al.*, 1999). A temperature sensitive mutant of SMC2, found in the budding yeast *Saccharomyces cerevisiae*, which facilitated defects in segregation in addition to partial decondensation of chromosomes led to the discovery of a gene essential for chromosome segregation (Strunnikov *et al.*, 1995). Additional studies determined that the non-SMC subunits are required for condensation, segregation, and more importantly cell viability (Lavoie *et al.*, 2002). Mutation of YCS4, the *S. cerevisiae* homolog to *Xenopus* XCAP-D2, leads to defects in rDNA condensation and segregation and is necessary for localizing topoisomerase I and II to chromosomes (Bhalla *et al.*, 2002). Loss of barren, the *Drosophila* homolog to CAP-H is responsible for abnormal chromosome segregation and subsequent lagging chromatids (Bhat *et al.*, 1996; Hirano *et al.*, 1997). These observations for critical subunit function extend into human condensin I as studies have determined that CAP-D2 is required for CAP-H localization to chromosomes and loss of CAP-D2 facilitates chromosomal misalignment during metaphase and subsequent delayed anaphase (Watrin and Legagneux, 2005). In all the preceding studies, a critical role for all five condensin I subunits is well established.

Regulation of condensin I function by phosphorylation

Condensin I function has been studied at length utilizing *Xenopus* egg extracts and the condensin I complex has been determined to bind to chromosomes specifically during mitosis. In addition to determining the necessity of condensin I for DNA condensation, it was also hypothesized that localization of the CAP-C and CAP-E subunits was regulated by mitotic-specific phosphorylation of CAP-G, CAP-D2, and CAP-H (Hirano *et al.*, 1997). As described previously, condensin I facilitates positive supercoiling in DNA when ATP and topoisomerase I are present. Adding even more complexity to the study of condensin I regulation is that CAP-G, CAP-D2, and CAP-H

are phosphorylated in a mitotic specific manner by Cdc2 which regulates the positive supercoiling activity of condensin I (Hirano *et al.*, 1997; Kimura *et al.*, 1998; Kimura and Hirano, 2000). Loss of Cdc2 is directly correlated with loss of condensin I activity as well as reduced phosphorylation of the three non-SMC subunits in *Xenopus* extracts; Comparable results were seen when this was repeated in human extracts (Kimura *et al.*, 1998; Kimura *et al.*, 2001). Despite the importance of Cdc2 phosphorylation of CAP-G, CAP-D2, and CAP-H in condensin I regulation, the specific phosphorylation sites for each subunit, except for those in XCAP-D2, have not yet been determined. More recently, it was hypothesized that Cdc2 phosphorylation plays a role in the actual binding of condensin I to chromosomes with an estimated 50% of the chromosomal localization of condensin I dependent on the Cdc2 kinase (Takemoto *et al.*, 2007).

Human disease and condensin

Increasingly, studies are focusing on the human health element associated with abnormal condensin I function. DNA condensation is essential for formation of individual chromosomes which are later segregated during anaphase. A correlation has been observed between premature chromosome condensation (PCC), microcephaly, and severe mental retardation (Neitzel *et al.*, 2002). A patient study from the same group later determined that autosomal recessive primary microcephaly (MCPH) is caused by a mutation in the MCPH1 gene that encodes the protein microcephalin which is responsible for negatively regulating condensin II in normal cells, thus preventing premature chromosome condensation (Trimborn *et al.*, 2006). MCPH is characterized by a significant reduction in brain size and mental retardation. Increasingly, studies are focusing on mutations of specific condensin subunits and their effect on disease progression. Mutations in the hCAP-C and hCAP-E subunits of condensin I have been isolated in patient cells with a non-Hodgkin's lymphoma known as pyothorax-associated lymphoma (PAL) which develops in the pleural cavity of patients (Ham *et al.*, 2007). A defect in a condensin II subunit, kleisin β is responsible for a defect in T cell development. These studies have indicated a clear role of condensin in human health conditions and a need for future research to expand this area. Current and future studies on condensin and its subunits appear to be focusing more in the direction of subunit

mutations in the enzyme which facilitate certain human defects and human diseases which occur as a result of abnormal regulation of condensin.

PRC1

Mitosis is an intricately regulated series of events leading to the division of a cell into two identical daughter cells in a process known as cytokinesis. Many facets of the cell cycle and mitosis such as DNA condensation are rather well characterized, although the process of cytokinesis and the proteins responsible for this process are still relatively undefined. It is known that cells utilize a contractile ring that creates a cleavage furrow dividing the cell into two lobes (Glotzer, 2005). The position of this furrow is controlled by the mitotic spindle which is comprised of a set of anti-parallel microtubules that are bundled in anaphase (Glotzer, 2003). Spindle assembly is a critical aspect leading to cytokinesis and is characterized by chromosome movement onto kinetochore microtubules and bundling of the non-kinetochore microtubules (Glotzer, 2003). The central spindle is necessary for cytokinesis as is the PRC1 protein.

Protein regulating cytokinesis 1, PRC1, is a 71kDa protein that is expressed primarily in the S and G2/M phase of the cell cycle and is expressed in all human cell lines (Jiang *et al.*, 1998). In addition to associating with the mitotic spindle, PRC1 bundles microtubules, which is essential for formation of the spindle as well as the overall completion of cytokinesis (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). In characterizing PRC1, a 40% similarity to the budding yeast protein Ase1p was found in the coiled-coil domains of PRC1 (Jiang *et al.*, 1998). Ase1 is necessary for spindle assembly, elongation and disassembly and localizes to the anaphase spindle (Juang *et al.*, 1997). A PRC1 and Ase1p homolog, Ase1 was later discovered in fission yeast and is necessary for spindle midzone formation in addition to serving a regulatory role at the cytokinesis checkpoint that halts division when the cytokinesis is disturbed (Yamashita *et al.*, 2005). SPD-1, a *Caenorhabditis elegans* homolog to PRC1 was recently characterized and shares a conserved region with PRC1 in addition to causing spindle midzone disruptions when loss of function mutations were studied (Verbrugghe and White, 2004). Feo, *Drosophila* homolog to PRC1, is also essential for midzone

formation and cytokinesis (Verni *et al.*, 2004). The conserved domains and similar functions between these PRC1 homologues is indicative of the essential requirement of these proteins for spindle formation and the critical role that regulation and formation of the central spindle plays in the completion of cytokinesis.

In addition to the conserved coiled-coil domains, PRC1 possesses two Cdk phosphorylation consequence sites at threonine 470 and threonine 481. A likely conclusion is that PRC1 is phosphorylated in a cell cycle regulated manner given that expression of PRC1 peaks during mitosis. In fact, PRC1 phosphorylation is weak in G1 and S phase but reaches a peak during mitosis (Jiang *et al.*, 1998). PRC1 is normally localized to the microtubules during metaphase, becomes concentrated at the spindle midzone later in anaphase and is present at the cell midbody during telophase. Mutation of the two phospho-threonine sites alters the normal localization of PRC1 as it leads to extensive MT bundling at the spindle in prometaphase and eventually blocks progression thru mitosis (Mollinari *et al.*, 2002). A stalled completion of mitosis in the PRC1 phosphorylation mutant indicates a critical importance for phosphorylation in the overall regulation of PRC1.

Studies have utilized siRNA mediated knockdown of PRC1 or antibody depletion experiments to determine the outcome on cell division in mitosis. Results from these experiments are virtually identical with each other in that cells progress normally through mitosis with a severe defect seen in the spindle midzone in anaphase with the ultimate outcome being abnormal cytokinesis and the presence of binucleated cells (Mollinari *et al.*, 2002; Kurasawa *et al.*, 2004; Mollinari *et al.*, 2005). However, these studies reported no changes in chromosome segregation but this defect was observed in a later study where the authors reported that approximately 80% of the cells lacking PRC1 displayed abnormal chromosome dynamics and mislocalization early in mitosis (Zhu *et al.*, 2006).

PRC1 was determined to be essential for the completion of cytokinesis. A later study expanded the role of PRC1 and determined a new interacting partner for PRC1, a chromokinesin Kif4. Chromokinesins are motor proteins and are split into two subfamilies, the Kinesin-4 or the Kinesin 10 family; the Kinesin-4 family associates with chromosomes, the mitotic spindle as well as the central spindle while the Kinesin-10 family is present on chromosome arms and microtubules (Mazumdar and Misteli, 2005).

Kif4 is a kinesin-4 family member and is known to participate in chromosome condensation, segregation, and more importantly cytokinesis. PRC1 and Kif4 appear to co-localize with each other during late anaphase at the spindle midzone and also at the cell midbody during telophase and cytokinesis (Kurasawa *et al.*, 2004). PRC1 and Kif4 were found to be essential for Kif4 to shift from binding chromosomes to microtubules as well as the correct localization of PRC1 at the spindle, respectively (Kurasawa *et al.*, 2004). Additionally, this interaction was determined to be regulated by phosphorylation of PRC1. Prior to metaphase, PRC1 is phosphorylated and is unable to bind to microtubules (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). At the metaphase to anaphase transition, PRC1 becomes dephosphorylated and interacts with Kif4 which is responsible for translocating PRC1 onto microtubules where it plays a role in formation of the spindle midzone (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005).

The interaction between PRC1 and Kif4 represents a critical regulatory step necessary for the formation of the spindle midzone. An earlier study observed that a deletion mutant of PRC1 bound to the mitotic spindle, but was unable to bind to Microtubules, suggesting that PRC1 could potentially bind to other protein complexes during mitosis in addition to MT binding (Mollinari *et al.*, 2002). This proved to be true with additional research on PRC1 and its interacting partners. PRC1 was found to interact with and direct localization of MKLP1 and CENP-E, two motor proteins during late mitosis (Kurasawa *et al.*, 2004).

There is still speculation surrounding the kinase(s) which phosphorylate PRC1, although studies hypothesize that Cdc2 or Cdk1 is responsible (Mollinari *et al.*, 2002; Abe *et al.*, 2007; Neef *et al.*, 2007). Interestingly, PRC1 has also been found to associate with a number of mitotic kinases. PRC1 interacts with the MAPKK-like kinase TOPK, only when TOPK is phosphorylated, and TOPK is found to upregulate PRC1 phosphorylation at threonine 481 by enhancing the cdk1/cyclin B complex (Abe *et al.*, 2007). More recently, it was reported that PRC1 interacts with and acts as a docking site for the Plk1 kinase during mitosis with this interaction being critical for spindle formation and the completion of cytokinesis (Neef *et al.*, 2007). Additionally, PRC1 was also determined to interact with Kif14, and new mitotic kinesin as well as other kinesins, and direct their localization to the mitotic spindle (Gruneberg *et al.*, 2006).

The ever expanding role for PRC1 not only in spindle formation, but in the critical regulation of additional proteins necessary for spindle formation and completion of cytokinesis represents a new direction in PRC1 research to elucidate the regulatory role of this multi-faceted protein from the start of mitosis to the end of cytokinesis. In addition, the human health implications for PRC1 function are being mapped out with research utilizing cancer cell lines. A report utilizing MEFs and HeLa cells deficient in BRCA2, a tumor suppressor implicated in breast and ovarian cancers, determined that loss of BRCA2 disrupts cytokinesis as well as alterations in chromosome number (Daniels *et al.*, 2004). A direct link between PRC1 function and breast cancer was found in a study that utilized a variety of breast cancer cell lines and determined that PRC1 was overexpressed in breast cancer cells and determined that downregulating PRC1, by using siRNA-mediated knockdown, prohibited growth of these cells (Shimo *et al.*, 2007). Using microarray analysis, PRC1 was also found to be downregulated in breast and prostate cancer cell lines in which the breast cancer susceptibility gene BRCA1, was knocked down by siRNA (Bae *et al.*, 2005). Given the importance of PRC1 for progression of correct cytokinesis and the newly discovered link in regulation of breast and prostate cancer cell growth, the future of PRC1 research will hopefully elucidate the mechanism for PRC1 and its involvement in carcinogenesis.

HSF2

HSF2 is one of four vertebrate heat shock transcription factors (HSFs) which transcriptionally regulate heat shock protein (hsp) gene expression and possess a conserved DNA binding domain (DBD) and oligomerization domain. HSF2 is 35% identical to HSF1 which is known to be the classical transcriptional regulator of the heat shock response (Pirkkala *et al.*, 2001). HSF1 is activated in response to stress, whether environmental or physiological, trimerizes and localizes to the nucleus where it binds to heat shock elements (HSEs) in the hsp70i gene promoter. HSF2 also upregulates hsp70 gene expression, although not as effectively as HSF1. HSF2 is known to be active during development, primarily in embryogenesis and spermatogenesis. Loss of HSF2 is not embryonic lethal although there are observed defects in spermatogenesis in males as well as abnormal egg production and reduced ovarian follicles in the females (Kallio *et al.*,

2002). Characterization of HSF^{-/-} MEFs revealed that these cells possess altered localization of HSF1 and hsp70 protein as well as defects in actin and the fibronectin network (Paslaru *et al.*, 2003). Interestingly, in addition to these described defects, there were also defects seen in cell morphology, as loss of HSF2 affected cell division as binucleated cells were present.

HSF2 is SUMO-1 modified which regulates the DNA binding of this transcription factor. This same study determined that HSF2 colocalizes with SUMO-1 in nuclear granules which are PML bodies (Goodson *et al.*, 2001). HSF2 also interacts with the PR65 subunit of PP2A, protein phosphatase 2A, and also regulates PP2A activity (Hong and Sarge, 1999). The regulation of PP2A by HSF2 was expanded when it was discovered that HSF2 participates in gene bookmarking at the hsp70 promoter.

Gene bookmarking is an epigenetic phenomenon in which certain gene promoters remain uncompact during mitosis in contrast to the majority of genomic DNA (Martinez-Balbas *et al.*, 1995; Michelotti *et al.*, 1997; John and Workman, 1998; Christova and Oelgeschlager, 2002). HSF2 binds to HSEs in the hsp70 and other HSE containing promoters such as hsp90, hsp27, and c-fos during mitosis and is important in regulating their expression as determined using siRNA-mediated knockdown of HSF2 (Wilkerson *et al.*, 2007). HSF2 recruits PP2A to the hsp70 gene promoter where PP2A then desphosphorylates the CAP-G subunit of the condensin I enzyme, responsible for DNA compaction early in mitosis, thereby inactivating condensin complexes present at the promoter and ultimately reducing compaction at the promoter in contrast to most genomic DNA (Figure 1.2) (Xing *et al.*, 2005). The reduced state of compaction at the promoter allows transcriptional machinery to be assembled rapidly on the promoter at the onset of G1 and ensures the cell's ability to induce the expression of cytoprotective hsp70 protein in response to stresses (Sarge and Park-Sarge, 2005). It was recently discovered that hsp70 (hspa1b) gene bookmarking also occurs in epididymal spermatozoa of mice as HSF2 as well as HSF1 are present at this promoter suggesting that these two transcription factors act collectively to regulate gene bookmarking during spermatogenesis (Wilkerson *et al.*, 2008).

Figure 1.1 Condensin enzyme from (Hagstrom and Meyer, 2003).

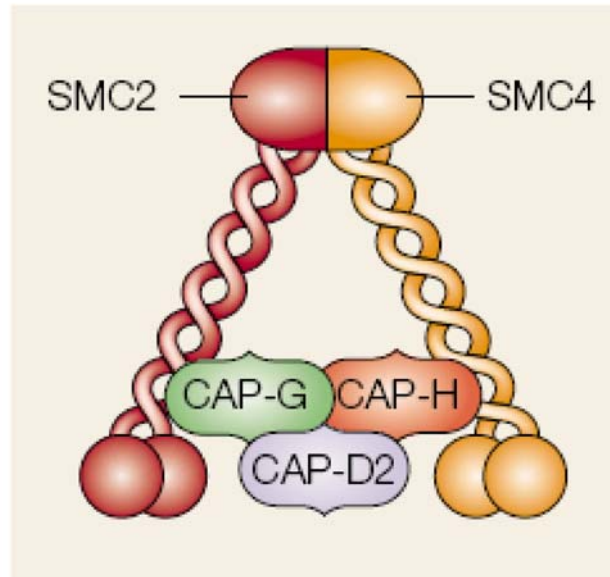


Figure 1.1 Schematic of condensin enzyme. Condensin is composed of five subunits. SMC2 (CAP-E) and SMC4 (CAP-C) form a heterodimer. The non-SMC subunits, CAP-G, CAP-H, and CAP-D2 associate with the SMC2/SMC4 heterodimer. From the beginning of mitosis to anaphase, the condensin enzyme complex is present on chromosomes when they are in a condensed form.

Figure 1.2 Model for hsp70 gene bookmarking from (Sarge and Park-Sarge, 2005).

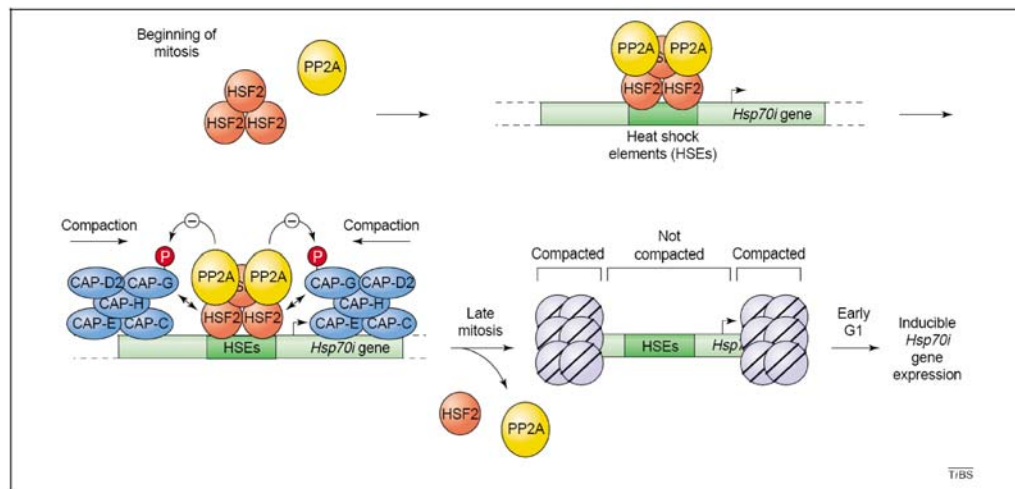


Figure 1.2 HSF2 gene bookmarking. The trimeric, DNA binding form, of HSF2 binds to the heat shock elements in the hsp70 gene promoter and recruits and interacts with protein phosphatase 2A (PP2A). HSF2 also interacts with the CAP-G subunit of condensin and this interaction promotes dephosphorylation of CAP-G by PP2A. This inactivates the condensin complex at this gene promoter and leaves this region of DNA open. During G1 after mitosis, the uncompact state at the promoter allows the transcriptional machinery to quickly assemble and induce expression of the cytoprotective hsp70 protein.

Chapter Two

Phosphorylation of CAP-G is required for its chromosomal DNA localization and proper chromosome morphology during mitosis

Goals of this study

Condensin activity is critical for DNA compaction early in mitosis. Various studies in *Xenopus* and human extracts have determined that the non-SMC subunits of condensin, CAP-G, CAP-D2, and CAP-H are phosphorylated by Cdc2 in a mitotic specific manner which regulates the positive supercoiling activity of condensin I as well as the localization of condensin to chromosomes (Hirano *et al.*, 1997; Kimura and Hirano, 1997; Kimura *et al.*, 1998; Kimura and Hirano, 2000; Takemoto *et al.*, 2007). In *Xenopus* studies, loss of Cdc2 reduced overall phosphorylation of these subunits and inhibited condensation activity (Kimura *et al.*, 1998). Both Cdc2 and Aurora B kinases are known to be active in mitosis and to phosphorylate condensin. However, a recent study indicated that both Cdc2 and Aurora B regulate chromosome binding of condensin during mitosis with the majority of chromosome localization dependent on Cdc2 (Takemoto *et al.*, 2007).

A previous study in *Xenopus* determined putative Cdc2 phosphorylation sites for XCAP-D2 (Kimura *et al.*, 1998). However, the specific phosphorylation sites for the remaining non-SMC subunits have not yet been elucidated and the regulation of these individual phospho-sites to overall condensin function is relatively unknown. Given the known importance of overall Cdc2 phosphorylation in regulation of condensin activity and regulation, the goal of this study was to determine the Cdc2 phosphorylation sites in human CAP-G (hCAP-G) and determine the significance of this phosphorylation on localization of the hCAP-G subunit as well as the overall effect to condensin activity during mitosis.

Materials and Methods

Peptide array analysis

To determine potential Cdc2 phosphorylation sites in CAP-G, peptide array analysis was utilized (Jerini, Inc.) In brief, an overlapping array of peptides representing the complete CAP-G sequence was spotted on a solid support and then incubated with Cdc2/cyclin B (NEB) in the presence of γ -³²P-ATP.

Plasmids/Antibodies

A human CAP-G cDNA (GenBank accession # AF331796) was directionally subcloned into the XhoI/KpnI multi-cloning site of pEGFP C2 vector (Clontech) to create an N-terminal green fluorescent protein (GFP) fusion to hCAP-G. To generate threonine to alanine mutants, site-directed mutagenesis was performed according to the manufacturer's instructions (Stratagene). Constructs were verified by sequencing (Davis Sequencing).

Enrichment of mitotic cell populations/Transient transfection of HeLa cells

HeLa ATCC cells were treated with 250ng/mL nocodazole, as determined by previous flow cytometry analysis to obtain mitotic indices of greater than 80%, (Sigma-Aldrich) for 18 hours for immunofluorescence analysis of mitotic cells or 500ng/mL for 6 hours for preparation of chromosome spreads for immunofluorescence (Watrin and Legagneux, 2005). For transfections, cells were transfected with pEGFP-CAP-G, pEGFP-CAP-G^{T308A}, pEGFP-CAP-G^{T332A}, pEGFP-CAP-G^{T931A}, or pEGFP-CAP-G^{T308A/T332A} jetPEI (Bridge Bioscience) according to the manufacturer's instructions. HeLa cells were grown in DMEM, 10% fetal bovine serum (FBS), 50 μ g/ml gentamicin at 37°C with 5% CO₂.

Western blot analysis

HeLa ATCC cells were transfected as described above followed by lysis in a buffer composed of 25% v/v glycerol, 420mM NaCl, 1.5mM MgCl₂, 0.2mM EDTA, and 20mM HEPES (pH 7.9) plus 1mM PMSF, 1x protease inhibitors (Roche), and 1mM DTT.

Lysates were resolved on 8% SDS-PAGE and western blotted with GFP mouse monoclonal antibody JL-8 (Clontech) and mouse monoclonal β -actin antibody (Sigma).

Fluorescence analysis

HeLa ATCC cells were seeded onto coverslips that were acid-washed and flamed, and then coated with laminin (5 μ g/mL) (Sigma). Cells were transfected and treated with nocodazole as described above. Cells were washed once in ice-cold 1xPBS, followed by fixation in 4% paraformaldehyde and permeabilization in 0.5% Triton X-100. After washes in 1xPBS, coverslips were mounted onto slides with Vectashield mounting medium plus 1.5 μ g/mL DAPI (4', 6 diamidino-2-phenylindole) (Vector Laboratories). The GFP-CAP-G constructs were visualized using a Nikon Eclipse 600 microscope with a 100x oil immersion objective and a Spot Camera with Metamorph imaging software.

Preparation of chromosome spreads

HeLa cells were transfected for 48 hours and subsequently treated with 500ng/mL nocodazole for 6 hours as described above. After treatment, cells were collected by shake-off and incubated with 75mM KCl for 30 minutes at 37°C and chromosome spreads were prepared by vertically dropping the cell suspension onto a slide using a pasteur pipet (Ono *et al.*, 2003; Mazumdar *et al.*, 2004). Slides were air-dried briefly and were then subjected to immunofluorescence analysis as described above.

Results

Cdc2 phosphorylation sites in CAP-G

In order to obtain a better understanding of CAP-G phosphorylation during mitosis, a peptide array analysis was utilized to determine potential Cdc2 sites within the protein. An overlapping array of peptides representing the full length sequence of the CAP-G protein was spotted onto a solid support and incubated with Cdc2/cyclin B (NEB) in the presence of γ -³²P-ATP. The results in (Figure 2.1A) indicate that there are three potential Cdc2 phosphorylation sites. The overlapping phosphopeptide set marked 1, 2, 3

correspond to a candidate Cdc2 site at Threonine 931, set 4 corresponds to another at Threonine 332, and the phosphopeptide marked 5 represents a site at Threonine 308. (Figure 2.1B) represents a schematic of the CAP-G protein showing the position of these three potential Cdc2 phosphorylation sites. Figure 2.1C illustrates conservation of these phosphorylation sites between species. Threonine 332 is well conserved among species whereas threonine 931 is absent in *Xenopus tropicalis*, *Xenopus laevis*, *Drosophila melanogaster*, and *Saccharomyces cerevisiae*. Serine or Threonine 308 is conserved among all species with the exception of *S. cerevisiae*.

Localization of CAP-G phosphorylation mutants during mitosis

In fission yeast, Cdc2 phosphorylation is responsible for the nuclear localization of condensin in mitosis (Sutani *et al.*, 1999). Therefore, we hypothesized that altering the putative Cdc2 phosphorylation sites in hCAP-G would affect the localization of this subunit with DNA during mitosis. To determine if the Cdc2 phosphorylation plays a role in regulating the localization of CAP-G with DN during mitosis, site-directed mutagenesis was used to mutate the threonine sites to alanine. Mutation of threonine residues to alanine is a common mutation to abolish threonine phosphorylation. HeLa cells were transfected with pEGFP-CAP-G, pEGFP-CAP-G^{T308A}, pEGFP-CAP-G^{T332A}, pEGFP-CAP-G^{T931A}, pEGFP-CAP-G^{T308A/T332A}, pEGFP-CAP-G^{T308A/T931A}, and pEGFP-CAP-G^{T332A/T931A} and then treated with nocodazole to enrich for mitotic cells. Figure 2.2 represents is a GFP western blot comparing the expression levels of these transfected constructs. pEGFP-CAP-G^{T931A} expresses in a similar manner as pEGFP-CAP-G, while the single and double mutants have similar expression levels. A B-actin western blot was used as a loading control. The results of the fluorescence analysis are shown in (Figure 2.3 and Figure 2.4). Fluorescence analysis of the single mutation of threonine 931 (Figure 2.3) does not appear to affect the localization of CAP-G with the DNA during mitosis. Double mutation of threonine 308 and 931 and threonine 332 and 931 appear to have no effect on CAP-G localization (Figure 2.4). However, mutation of the single threonine 308 and threonine 332 (Figure 2.3) appears to alter the localization of CAP-G with the DNA during mitosis. Mutation of both threonines 308 and 332 (Figure 2.4) leads to a more drastic change in CAP-G localization with DNA during mitosis leading to

the conclusion that the Cdc2 phosphorylation of both threonines 308 and 332 is important for correct CAP-G localization in mitotic cells.

Cdc2 phosphorylation of CAP-G is important for DNA compaction during mitosis

Considering the role for Cdc2 phosphorylation in the localization of CAP-G during mitosis, we were interested in determining if the Cdc2 phosphorylation was also responsible for regulating the overall function of the condensin enzyme in DNA compaction during mitosis. To determine if CAP-G phosphorylation plays a role in regulating DNA compaction, an immunofluorescence analysis of mitotic chromosome spreads was utilized to determine if DNA compaction was altered versus the wild-type protein. To prepare the spreads, cells were enriched for mitotic cells with nocodazole treatment and incubated in a hypotonic solution. Following this incubation, spreads were prepared by vertically dropping the cell suspension onto a slide using a Pasteur pipet and subjected to immunofluorescence. Figures 2.5 and 2.6 illustrate the results of this experiment. As seen in fluorescence of mitotic cells, chromosome spreads prepared from cells transfected with the single CAP-G threonine to alanine mutant at amino acid 931 (Figure 2.5) do not appear to show any change in DNA compaction as evidenced by DAPI staining of DNA. Chromosomes overexpressing GFP-CAP-G^{T308A/T931A} and GFP-CAP-G^{T332A/T931A} (Figure 2.6) display little or no changes in chromosome compaction compared to wild-type (Figure 2.5). Spreads prepared from cells transfected with the single threonine 308 or 332 mutant are much more difficult to distinguish individual chromosomes and appear tangled with each other and are less condensed when compared to the wild type protein (Figure 2.5). Additionally, chromosomes expressing the GFP-CAP-G^{T308A/T332A} protein take on a fuzzy and more dispersed appearance than the cells expressing the CAP-G wild type protein (Figure 2.6).

Discussion and Future Directions

Cdc2 phosphorylation of the non-SMC subunits during mitosis regulates the positive supercoiling activity of condensin I as well as condensin localization on chromosomes (Hirano *et al.*, 1997; Kimura and Hirano, 1997; Kimura *et al.*, 1998;

Kimura and Hirano, 2000; Takemoto *et al.*, 2007). Given the importance of the overall Cdc2 phosphorylation of the non-SMC subunits to condensin function, the specific phosphorylation sites of CAP-G and CAP-H have not been determined, although the putative Cdc2 sites have been mapped for *Xenopus* CAP-D2 (Kimura *et al.*, 1998). This study has determined that there are three putative Cdc2 sites at threonines 308, 332, and 931 for the hCAP-G condensin subunit. Loss of the threonine 308 and 332 phosphorylation sites appears to correlate with altered localization of CAP-G with the DNA in mitosis as well as changes in chromosome compaction of cells expressing phosphorylation site mutants. Mutation of threonine to alanine is commonly used to abolish phosphorylation of threonine sites.

Previous studies investigated an epigenetic phenomenon known as gene bookmarking, in which specific gene promoters remain relatively uncompact in comparison to most genomic DNA (Martinez-Balbas *et al.*, 1995; Michelotti *et al.*, 1997; John and Workman, 1998; Christova and Oelgeschlager, 2002). A subsequent study defined the mechanism by which heat shock transcription factor 2 (HSF2) mediates gene bookmarking at the hsp70 promoter, one of the promoters known to be involved in this phenomenon. HSF2 binds to the heat shock elements (HSEs) in hsp70 and other heat shock gene promoters during mitosis and recruits the phosphatase PP2A, while simultaneously interacting with the CAP-G subunit of human condensin (Xing *et al.*, 2005). This interaction leads to the dephosphorylation/inactivation of CAP-G and the condensin complex and reduces compaction at this specific region of chromosomal DNA. The reduced compaction at the hsp70 promoter allows rapid reassembly to a transcriptionally competent state in early G1 phase of the cell cycle and ensures the ability of the cell to induce this protective heat shock protein if stress conditions occur (Sarge and Park-Sarge, 2005). Further strengthening the critical role for CAP-G in hsp70 bookmarking, this study has determined the specific Cdc2 phosphorylation sites for CAP-G and established their functional role in regulating CAP-G localization and overall DNA compaction state during mitosis.

DNA condensation represents a critical step at the beginning of mitosis and allows proper segregation of chromosomes which is essential for the formation of two daughter cells in cytokinesis each with an identical set of replicated DNA. Failure to

correctly condense and segregate chromosomes can lead to aneuploidy characterized by abnormal chromosome number and is linked to birth defects and cancer (Legagneux *et al.*, 2004). In our CAP-G phosphorylation mutants, individual chromosomes were less defined when compared to wild type CAP-G. A similar defect on chromosomes was observed in separate studies when CAP-G and CAP-D2 were knocked down using siRNA (Ono *et al.*, 2003; Watrin and Legagneux, 2005). Although the present study did not address specific changes in condensin activity, a supercoiling assay using cell extracts from cells transfected with the CAP-G^{T308A}, CAP-G^{T332A}, and CAP-G^{T308A/T332A} would determine if these two phosphosites inhibit condensin activity when Cdc2 phosphorylation is abolished. When DNA condensation and segregation is disrupted, aneuploidy can occur. While the chromosome spreads in this illustrated that there was a change in DNA compaction in the phosphosite mutants, the question of whether inhibiting CAP-G phosphorylation facilitates aneuploidy has yet to be answered. A study previously determined that depletion of CAP-G alters kinetochore structure and ultimately leads to abnormal chromosomal segregation and alignment (Ono *et al.*, 2004). siRNA mediated knockdown of another non-SMC subunit, CAP-D2, has been shown to increase the number of cells present in prometaphase and metaphase while cells in the later stages of anaphase and telophase decreased over time (Watrin and Legagneux, 2005). Flow cytometry analysis of cells overexpressing the CAP-G phosphosite mutants could potentially answer the question of whether Cdc2 phosphorylation of CAP-G is responsible for mitotic progression and potentially chromosome segregation. Given that phosphorylation of the three non-SMC subunits has been shown to regulate overall condensin enzymatic activity, the possibility exists that CAP-G phosphorylation, as well as all non-SMC subunit phosphorylation, plays a regulatory role in the progression of mitosis. Since Cdc2 phosphorylation appears to be important for regulating CAP-G localization in addition to regulating condensin function in mitosis, future studies to determine the exact Cdc2 sites for the remaining non-SMC subunits will be necessary to determine the regulatory role for this phosphorylation and the effect on DNA condensation, chromosome segregation and alignment, as well as overall progression through mitosis.

Figure 2.1A and B. Identification of the Cdc2 phosphorylation sites in the CAP-G condensin subunit.

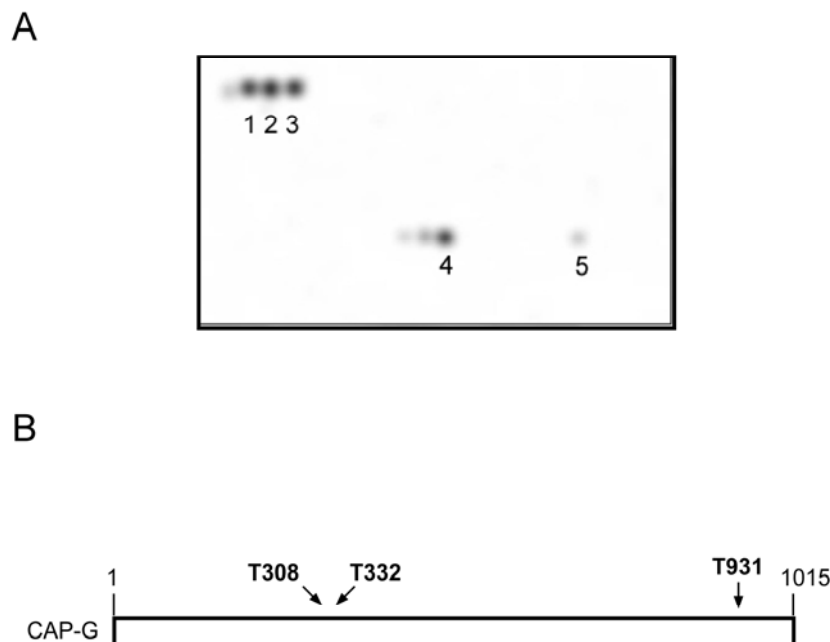


Figure 2.1A and 2.1B. Identification of Cdc2 phosphorylation sites in hCAP-G. To identify Cdc2 phosphorylation sites for CAP-G, an overlapping array of peptides representing the complete CAP-G sequence was spotted on a solid support and then incubated with Cdc2/cyclin B (NEB) in the presence of γ - ^{32}P -ATP. (A) The results of phosphopeptide analysis showing three potential Cdc2/cyclin B phosphorylation sites in the CAP-G protein are shown (assay by Jerini, Inc.). The overlapping phosphopeptide set marked 1, 2, 3 corresponds to a candidate Cdc2 site at threonine 931, set 4 corresponds to another at Threonine 332, and the phosphopeptide marked 5 represents a site at Threonine 308. (B) Schematic showing the location of these three candidate phosphorylation sites within the CAP-G protein.

Figure 2.1C. Alignment and conservation of the Cdc2 phosphorylation sites in the CAP-G condensin subunit among different species.

C

	308	332	931
<i>H. sapiens</i>	ALFSI T PLSEL	PVETL T PEIAL	KEVYM T PLRGV
<i>M. musculus</i>	SLFSMTPLSEL	PVETLTPEIAL	---YMT P VRDG
<i>P. troglodytes</i>	ALFSI T PLSEL	PVETLTPEIAL	KEVYM T PLRGV
<i>R. norvegicus</i>	SLFSMSPLSEL	PVETLTPEIAL	---YRT P ARNV
<i>B. taurus</i>	ALFSMTPLNEL	PADTLTPEFAL	KDVYI T PVKEV
<i>X. tropicalis</i>	ALFSVSPVSEL	PVETLT P ENVL	No sig. similarity
<i>X. laevis</i>	ALFSVSPVGEL	PVETLT P ENVL	KQKDEANCDEN
<i>D. melanogaster</i>	VIFEQ T DIQQL	PHDSV T VELLL	DVTQSA A ITAV
<i>S. cerevisiae</i>	ALFQSR P --DI	--KDF T VEIAF	No. sig. similarity

Figure 2.1C. Conservation of CAP-G Cdc2 phosphorylation sites. (C) Conservation of threonine 308, 332, and 931 phosphorylation sites among species compared to *Homo sapiens* CAP-G (Accession number AF331796).

Figure 2.2 Western blot of GFP-CAP-G transfection levels.

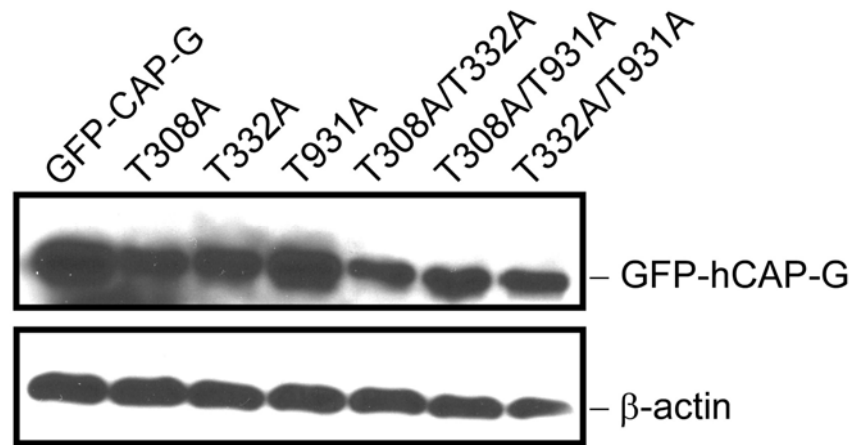


Figure 2.2 Overexpression levels of GFP-CAP-G mutants. HeLa cells were transfected with GFP-hCAP-G, GFP-hCAP-G^{T308A}, GFP-hCAP-G^{T332A}, GFP-hCAP-G^{T931A}, GFP-hCAP-G^{T308A/T332A}, GFP-hCAP-G^{T308A/T931A}, GFP-hCAP-G^{T332A/T931A} and nocodazole treated to enrich for mitotic cell populations. Lysates were western blotted to detect for GFP-CAP-G using GFP monoclonal antibodies (Clontech). Lysates were probed with β-actin as a loading control.

Figure 2.3 Threonines 308 and 332 are important for CAP-G localization during mitosis.

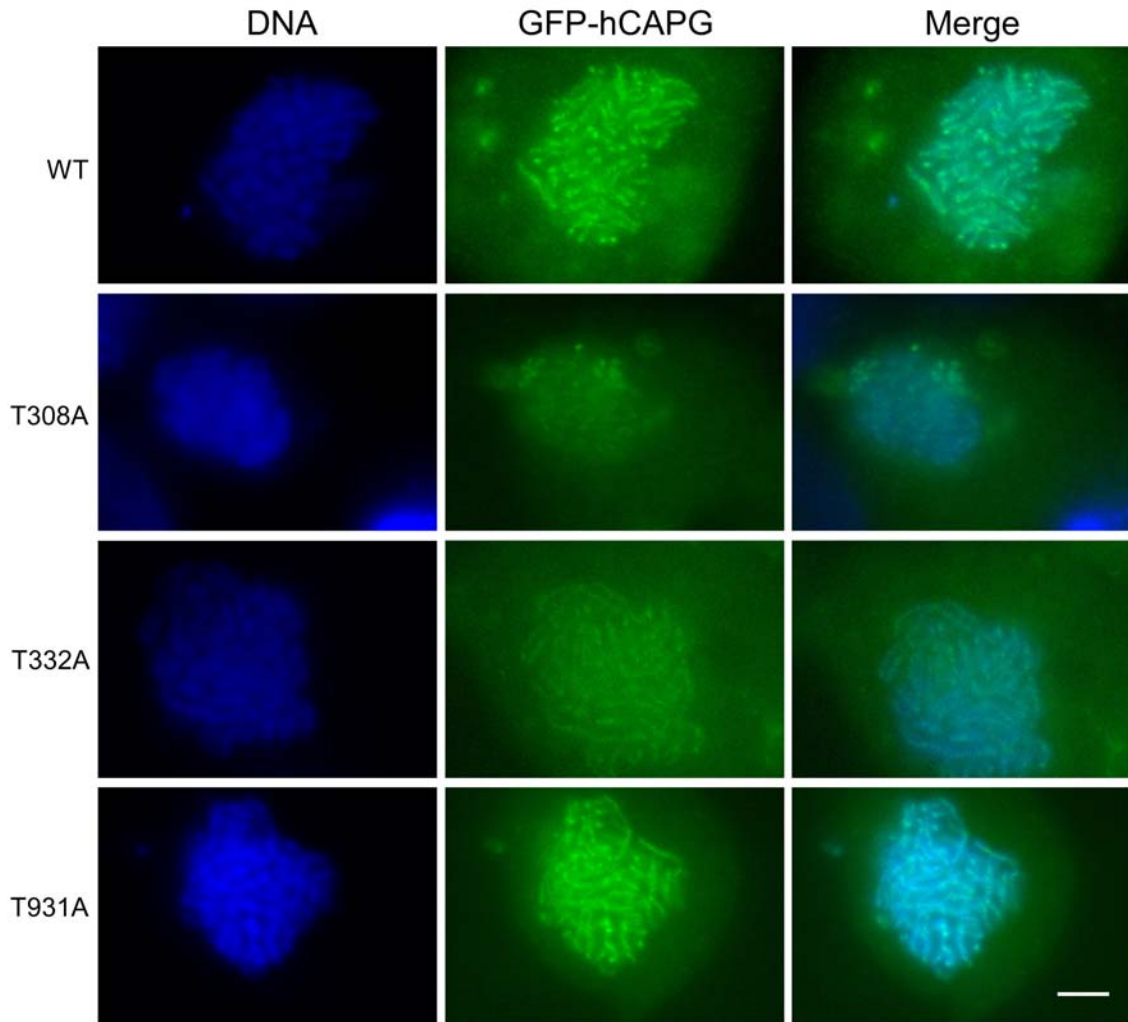


Figure 2.3 Fluorescence analysis of CAP-G phosphosite mutants. HeLa cells were transfected with GFP-hCAP-G, GFP-hCAP-G^{T308A}, GFP-hCAP-G^{T332A}, GFP-hCAP-G^{T931A}, and nocodazole treated to enrich for mitotic cell populations. The cells were then subjected to fluorescence microscopy, with DAPI staining used to detect the DNA. A change in localization of CAP-G was based on co-staining with DNA compared to wild type GFP-CAP-G protein. Scale bar, 10 μ m.

Figure 2.4 Mutation of both threonine 308 and 332 alter localization of CAP-G with DNA.

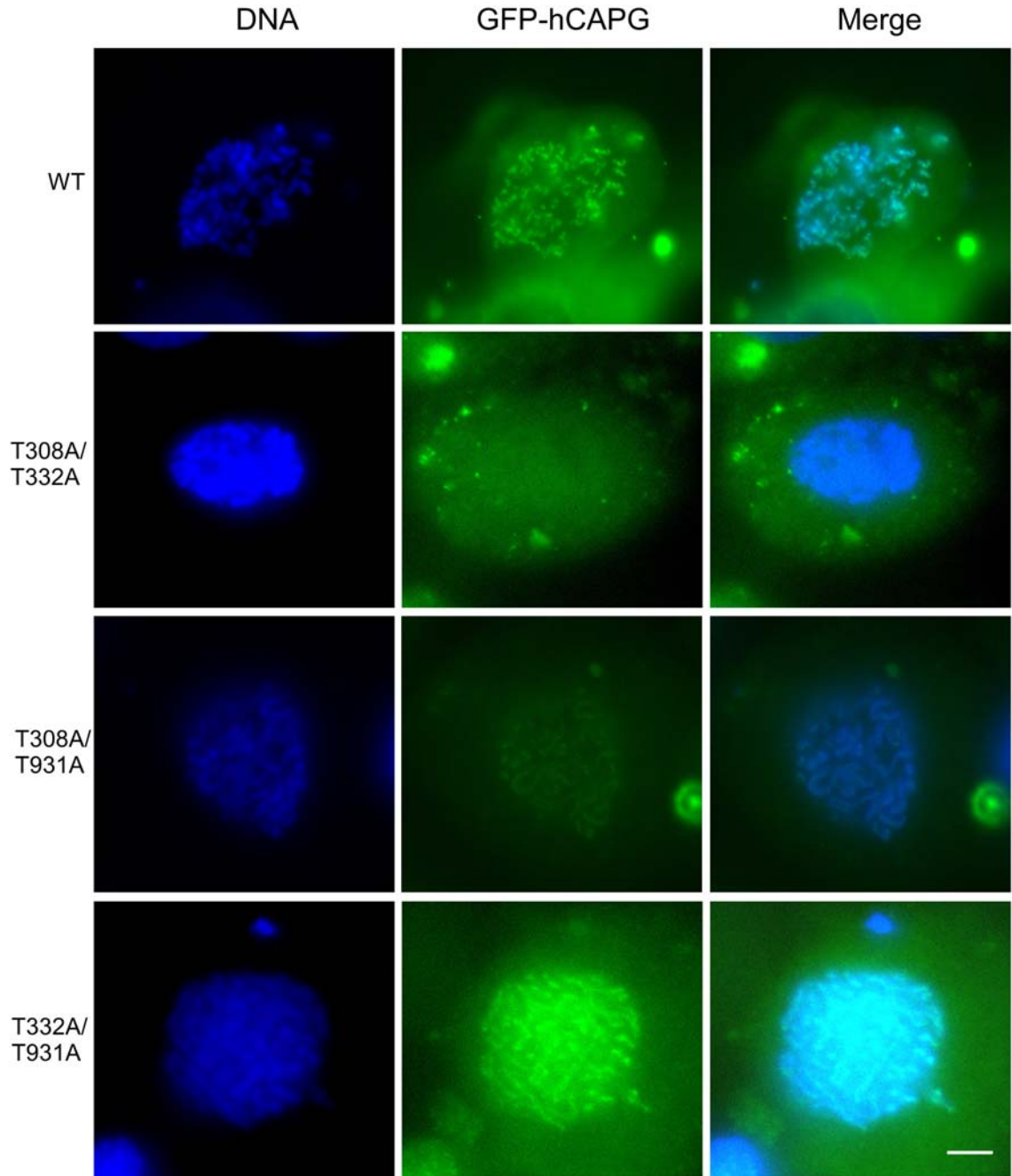


Figure 2.4 Fluorescence analysis of CAP-G double phosphosite mutants. HeLa cells were transfected with GFP-hCAP-G, GFP-hCAP-G^{T308A/T332A}, GFP-hCAP-G^{T308A/T931A}, and GFP-hCAP-G^{T332A/T931A}, followed by nocodazole treatment to enrich for mitotic cell populations. The cells were then subjected to fluorescence microscopy, with DAPI staining used to detect the DNA. Scale bar, 10µm.

Figure 2.5 CAP-G phosphorylation is important for chromosome morphology in mitosis.

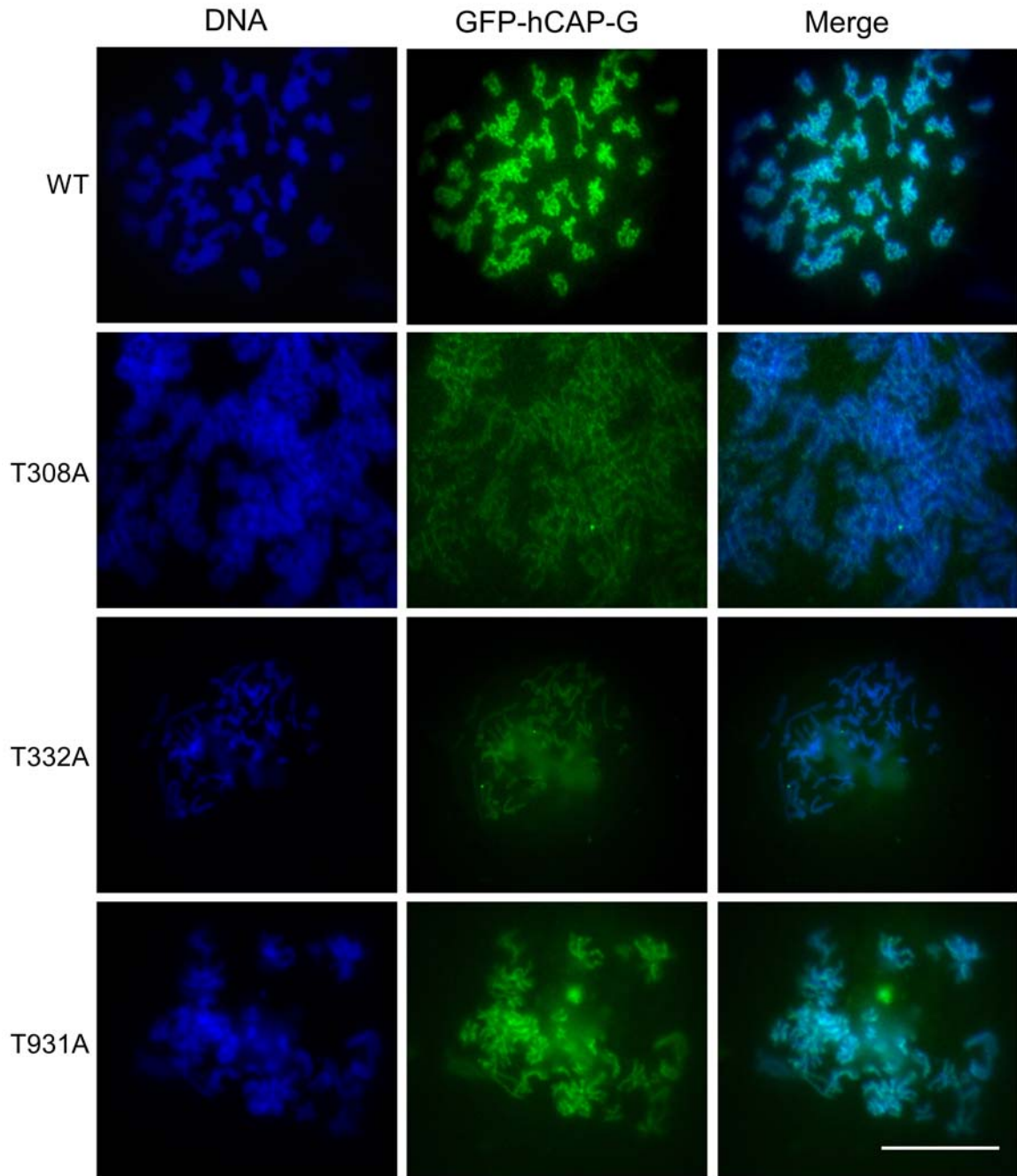


Figure 2.5 Fluorescence analysis of CAP-G single phosphosite mitotic chromosome spreads. HeLa cells were transfected with GFP-hCAP-G, GFP-hCAP-G^{T308A}, GFP-hCAP-G^{T332A}, and GFP-hCAP-G^{T931A} followed by nocodazole treatment to enrich for mitotic cells. The cells were then subjected to fluorescence microscopy, with DAPI staining used to detect the DNA. Scale bar, 10 μ m.

Figure 2.6 Threonines 308 and 332 are important for chromosome structure during mitosis.

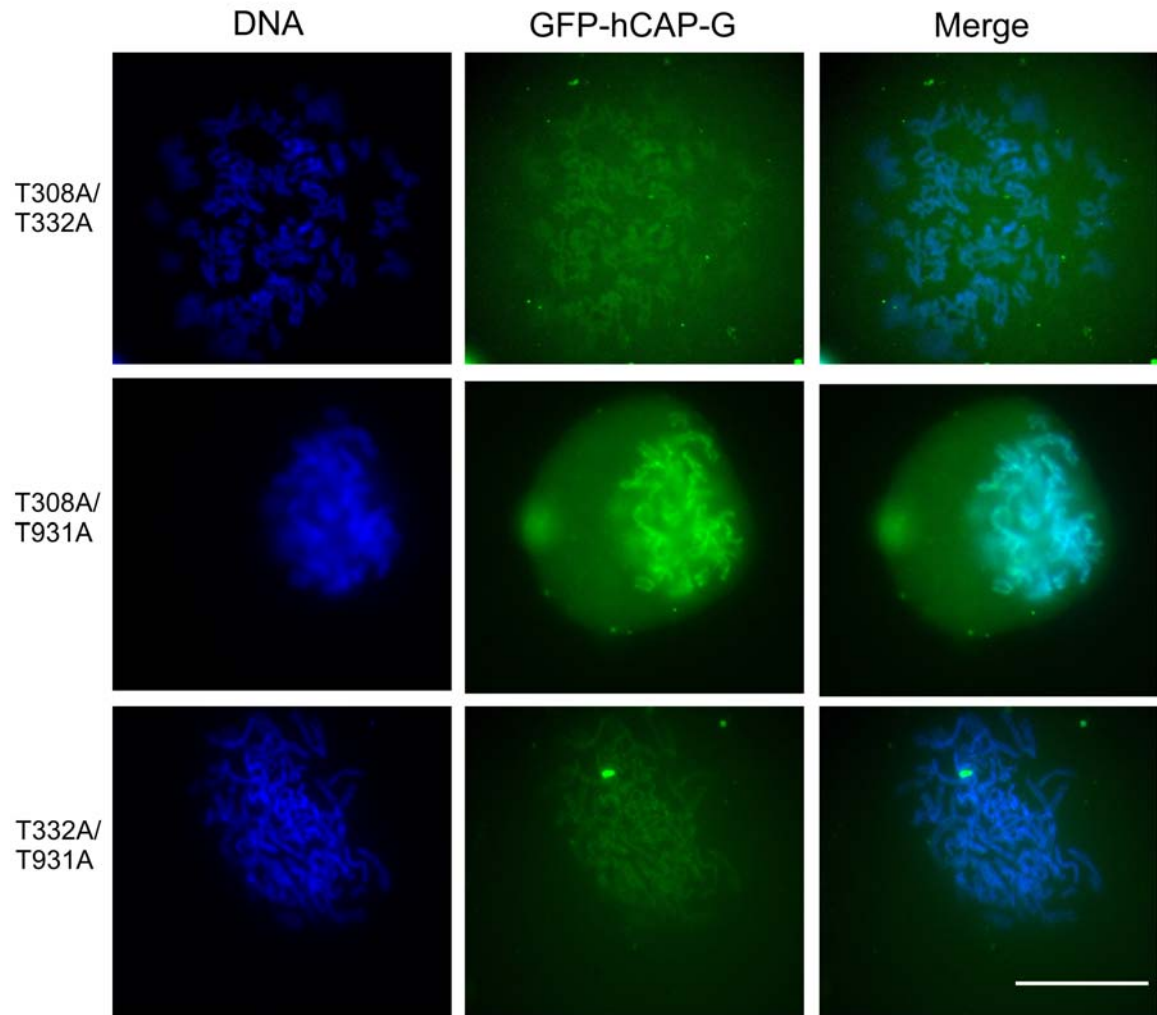


Figure 2.6 Fluorescence analysis of CAP-G double phosphosite mitotic chromosome spreads. HeLa cells were transfected with GFP-hCAP-G^{T308A/T332A}, GFP-hCAP-G^{T308A/T931A}, and GFP-hCAP-G^{T332A/T931A} followed by nocodazole treatment to enrich for mitotic cells. The cells were then subjected to fluorescence microscopy, with DAPI staining used to detect the DNA.

Chapter Three

PRC1 associates with the hsp70i promoter and interacts with HSF2 during mitosis

Goals of this study

The ultimate goal in mitosis is to correctly condense and segregate DNA so that during cytokinesis a cell divides into two identical daughter cells. In recent years, the study of the PRC1 protein has generated crucial insight into the role of this protein in the formation of the mitotic spindle as well as in proper chromosomal segregation and localization. Currently, studies are focusing on characterizing the interacting partners of PRC1 in mitosis that may further expand the role of PRC1 from that of a microtubule bundling protein located on the mitotic spindle to a protein critical in the regulation of mitosis and cytokinesis. An example is a recent study which outlined a mechanism in which PRC1 acts as a docking site for Plk1 in mitosis allowing Plk1 to change localization from the centrosome in metaphase to the kinetochore in anaphase (Neef *et al.*, 2007). An important mitotic specific function for HSF2 was discovered when HSF2 was found to bookmark the hsp70 gene promoter during mitosis through interaction with the PP2A phosphatase which inactivates the CAP-G subunit of condensin resulting in an uncompact promoter that quickly becomes transcriptionally competent in G1 allowing the cell to rapidly induce hsp70 in the presence of cell stress (Sarge and Park-Sarge, 2005; Xing *et al.*, 2005). Given the already established importance of PRC1 in mitosis and the critical role of HSF2 in gene bookmarking, the goal of this study was to characterize the interaction between these two proteins during mitosis and determine possible implications for this interaction in bookmarking and/or cytokinesis.

Materials and Methods

Plasmids/Antibodies

pOTB7 Plasmid containing full length PRC1 cDNA clone (MGC3669) was purchased from Invitrogen. The plasmid pEGFP-PRC1 was cloned using primers to add XhoI and EcoRI sites to the 5' and 3' ends of PRC1. Following digestion with XhoI and EcoRI, the insert was cloned into the XhoI and EcoRI sites of pEGFP-C2 (Clontech). Affinity purified antibodies synthesized against the peptides CSKASKSDATSGILNSTNIQS or CYLCELAPAPLDSDMPLLDS which correspond to the C-terminal residues 601 to 620 of PRC1 (Jiang *et al.*, 1998) 498 to 517 of mouse HSF2 (which is identical to the C-terminal sequence of human HSF2), respectively, are from Bethyl Laboratories, Inc.

Enrichment of mitotic cell populations/Transient transfection of HeLa cells

HeLa ATCC and Jurkat cells were treated with nocodazole (Sigma-Aldrich) at 250ng/mL for 18 hours or with 10nM Taxol (T7402 Sigma-Aldrich) for 24 hours (Woods *et al.*, 1995; Xing *et al.*, 2005; Niikura *et al.*, 2007). For transfections, cells were transfected with pEGFP HSF2 or pEGFP-PRC1 using Effectene (Qiagen) or jetPEI (Bridge Bioscience) according to the manufacturer's instructions. HeLa cells were grown in DMEM, 10% fetal bovine serum (FBS), 50 µg/ml gentamicin at 37°C with 5% CO₂. Jurkat cells were cultured in RPMI 1640, 10% FBS, and 50µg/ml gentamicin at 37°C with 5% CO₂.

Yeast two-hybrid analysis and β -galactosidase assay

An HSF2 or HSF1 "bait" construct consisting of full length HSF2 or HSF1 inserted in-frame into the vector pGBD-C1 was transformed into yeast strain pJ69-4A. The resulting strain was then transformed with a mouse whole embryo cDNA library (Hollenberg *et al.*, 1995). For the HSF2 deletion mutants, full length HSF2, HSF2 (1-473), HSF2 (1-387), HSF2 (1-281), and HSF2 (1-168) were transformed as above. To confirm the

interaction, the HSF2 constructs containing the partial mPRC1 cDNA, referred to as mPRC1 (118-233), were transformed back into yeast and the ability of HSF2 or HSF1 and the mPRC1 clone to interact was determined by growth on selective media lacking adenine or histidine. For assay of interaction strength using β -galactosidase activity, yeast extracts were incubated with Z Buffer (60mM Na_2HPO_4 and 40mM NaH_2PO_4 (pH 7.0), 10mM KCl, 1mM MgSO_4 , 50mM β -mercaptoethanol). After addition of 4mg/ml o-nitrophenyl- β -D-galactoside (ONPG) substrate, samples were incubated at 30°C for 30 minutes and then the OD_{420} was measured.

In vitro binding assay

pGEX-HSF2, pGEX-HSF1, or empty pGEX vector was transformed into Top10 E. coli strain and grown on selective media. An overnight 3ml culture was added to a one liter culture of LB/Amp and grown until the culture reached an OD_{600} of 0.600. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 1mM. After a four hour induction at 30°C, bacteria were centrifuged and washed with 1xPBS, quick frozen, and stored at -80°C. For experiments, a bacterial pellet representing a 50mL culture was prepared by resuspending in (1xPBS, 1mM PMSF, 1xprotease inhibitor, 1mM DTT, and 1.5% sarkosyl (for GST-HSF2)) and sonicating on ice, four rounds at 15 seconds each at 50%. GST-HSF2, GST-HSF1, and GST expressed in E. coli were bound to glutathione-agarose beads and then incubated with ^{35}S -labeled in vitro translated full length in 0.5 ml of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 1mM PMSF, and 1X protease inhibitor (Roche) for 16 hours at 4°C. The beads containing bound proteins were then washed four times at 4°C with 10 mM Tris-HCl (pH 7.0), 1% Triton X-100, 150 mM NaCl, 2 mM DTT, 1 mM PMSF. The beads were then resuspended in 20 μl SDS-PAGE loading buffer, boiled for 5 minutes and then subjected to SDS-PAGE on a 10% gel. The gels were then dried and exposed to X-ray film to detect the ^{35}S -labeled in vitro translated PRC1 proteins. The amounts of GST-HSF2, GST-HSF1, and GST proteins bound to the beads were determined by SDS-PAGE followed by Western blot using goat polyclonal anti-GST antibody (Amersham). For experiments using HeLa ATCC, mitotic cells were lysed in 10mM Tris-HCl (pH 7.0),

1% Triton X-100, 150mM NaCl, 2mM DTT, 1mM PMSF and incubated and washed as described above. The amount of bound PRC1 was determined by SDS-PAGE followed by Western blot using goat polyclonal anti-PRC1 antibody (Bethyl).

Immunoprecipitation analysis

Nocodazole or taxol treated HeLa ATCC cells were lysed in 25 mM HEPES, 100 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM β -glycerophosphate, 20 mM paranitrophenylphosphate, 100 μ M sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1x Complete mini-protease inhibitor cocktail [Roche Diagnostics]) supplemented with 20 mM N-ethylmaleimide and 5 μ M MG132, followed by centrifugation for 25 min at 15,000 x g at 4°C (Hietakangas *et al.*, 2003). Lysates were precleared with 4 μ g rabbit or goat IgG and 40 μ l of a 50% slurry of Protein G Sepharose in supplemented TEG buffer (20 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol) containing 150 mM NaCl and 0.1% Triton X-100 for 1 hour at 4°C. Samples were incubated with 3 μ L anti-HSF2 rabbit polyclonal or 4 μ g anti-PRC1 goat antibody for 30 minutes at room temperature followed by incubation with 40 μ l of Protein G Sepharose overnight at 4°C. Beads were washed with supplemented TEG buffer and resuspended in 20 μ L SDS loading buffer. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blot according to established lab protocols using anti-PRC1 antibody (Bethyl) or GFP mouse monoclonal antibody(Clontech) (Goodson *et al.*, 2001).

Immunofluorescence analysis

HeLa ATCC cells were seeded onto coverslips that were acid-washed and flamed, and then coated with laminin (5 μ g/mL) (Sigma-Aldrich). Cells were washed once in ice-cold 1xPBS, followed by fixation in 4% paraformaldehyde and permeabilization in 0.5% Triton X-100/0.5% saponin. Cells were blocked in 5% bovine serum album followed by incubation with anti-HSF2 goat polyclonal antibody or anti- α -tubulin mouse monoclonal antibody clone B-5-1-2 (T5168 from Sigma-Aldrich) for 1 hour at 37°C. After multiple

washes with PBS, the coverslips were incubated as above with either Horse anti-mouse Texas Red (Vector Laboratories), Alexa Fluor® 488 donkey anti-goat IgG or Alexa Fluor® 594 donkey anti-goat IgG (Invitrogen).

After a final wash with PBS, coverslips were mounted on a slide with Vectashield mounting medium plus 1.5µg/mL DAPI (4', 6 diamidino-2-phenylindole) (Vector Laboratories). Immunostaining was visualized using a Nikon Eclipse 600 microscope with a 100x oil immersion objective and a Spot Camera with Metamorph imaging software.

Chromatin Immunoprecipitation (ChIP)

Jurkat cells were treated with nocodazole (250ng/mL for 18 hours) or Taxol (10nM for 24 hours) as indicated and subjected to ChIP analysis according to established protocols in our laboratory (Xing *et al.*, 2005) with the following modifications. Pre-cleared chromatin was incubated with 13µg of goat PRC1 antibodies or control goat IgG, and rotated at 4°C for 16 hours. DNA was purified using a QIAquick PCR Purification Kit (Qiagen Inc.) and eluted in 50µl of 10mM Tris (pH 8.5). Immunoprecipitated DNA and input samples obtained prior to immunoprecipitation were analyzed by traditional PCR and by quantitative real time PCR (QPCR). QPCR was performed with a Stratagene Mx 4000 system using Brilliant SYBR Green QPCR master mix (Stratagene). Samples were checked for specific amplification using dissociation curves analysis software. PCR products were also assayed on polyacrylamide gels with ethidium bromide staining to ensure they were of the expected size. The Ct values were normalized to input DNA (DNA before immunoprecipitation step) and IgG controls using the formula $2^{[(Ct\ IgG - Ct\ Input) - (Ct\ Ab - Ct\ Input)]}$ (where Ab=PRC1 antibodies or IgG). Data is represented as fold differences relative to the IgG antibody, which was set to 1. The data shown represents at least three independent ChIP assays. Error bars represent standard error of the means. Traditional PCR was performed using a Stratagene Robocycler. The linear range of amplification was identified by assaying a variety of cycle numbers. PCR products were resolved by polyacrylamide gel electrophoresis and DNA visualized with ethidium bromide staining. The following primers (shown 5' to 3') from Integrated DNA

Technologies (Coralville, IA) were used for all PCR reactions: hhsp70: (+) CAACACCCTTCCCACCGCCACTC, (-) CCAGCCTTCCTTGGACCAATCAG.

Results

Interaction between HSF2 and PRC1 is specific

In order to obtain a better understanding of the regulation and functions of HSF2 in cells, a yeast two-hybrid screen was performed using the HSF2 protein as the bait to identify protein partners of this HSF. One of the interacting clones identified by this screen represents a sequence in the amino terminal half of mouse PRC1, comprising amino acids 118-233, which are homologous to amino acids 118-233 of human PRC1 (Fig. 3.1A). Additionally, the *Mus musculus* PRC1 nucleotide and protein sequences from the yeast two-hybrid were aligned against *Homo sapiens* PRC1 and were found to be 82% identical to human PRC1 in both nucleotide and protein sequence (Figure 3.1B). As a control to test the specificity of this interaction, a yeast two-hybrid assay was performed using HSF1 as bait. Analysis of yeast two-hybrid selective growth and β -galactosidase activity data reveals that the interaction between PRC1 is specific for HSF2 as HSF1 does not appear to interact (Figure 3.2 and Figure 3.3). To determine the specific PRC1 interacting region in HSF2, a yeast two-hybrid screen was utilized with HSF2 deletion mutants as bait and the partial PRC1 as prey (Figure 3.4). To determine strength of interaction and the HSF2 interacting region for PRC1, a β -galactosidase assay with the HSF2 mutants was used. Full length HSF2 interacts with PRC1 around 16-fold higher than empty vector alone. Interaction between PRC1 and HSF2 became progressively weaker as the HSF2 protein was sequentially deleted (Figure 3.5). The biggest change came between HSF (1-281) and HSF2 (1-168) where the interaction strength decreased approximately 4-fold leading to the conclusion that the PRC1 interacting region in HSF2 is between amino acids 168-281 (Figure 3.6).

Recombinant HSF2 associates with in vitro translated PRC1 and endogenous PRC1 from mitotic cell lysates

To independently confirm this interaction between HSF2 and PRC1, and to test whether the interaction is direct, in vitro binding experiments were performed using ^{35}S -labeled in vitro translated human PRC1 that was expressed from a full-length cDNA clone (MGC3669). The ^{35}S -labeled PRC1 was incubated with GST-HSF2, or GST bound to glutathione agarose beads, washed, and then subjected to SDS-PAGE and autoradiography to determine the amount of ^{35}S -labeled PRC1 that was bound. The results of this experiment, shown in Figure 3.7, indicate that the in vitro translated PRC1 interacts with the GST-HSF2 but not GST alone, thereby supporting the yeast two-hybrid finding of a specific interaction between HSF2 and PRC1 and also suggesting that the interaction is direct.

Prior to performing an in vitro binding assay to determine if endogenous PRC1 can associate with HSF2, we characterized the PRC1 antibody (Clontech). Consistent with previously published figures using the antibody which recognizes amino acids 601 to 620 of human PRC1, our PRC1 antibody (Figure 3.8A) localizes comparably throughout mitosis (Jiang *et al.*, 1998). To test whether recombinant HSF2 could associate with endogenous mitotic PRC1, we incubated GST-HSF2, GST-HSF1 or GST bound to glutathione agarose beads with lysates of mitotic HeLa cells. Following washing, the amount of PRC1 from these extracts that remained bound was determined by SDS-PAGE and Western blot using antibodies against PRC1. The results indicate that endogenous PRC1 present in mitotic cell extracts is able to interact specifically with purified recombinant HSF2 (Figure 3.8B).

HSF2 and PRC1 interact during mitosis

PRC1 is a CDK substrate protein known to associate with the mitotic spindle during mitosis (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). Since PRC1 is known to be expressed predominantly during the S and G2/M phase of the cell cycle, and to function during mitosis (Jiang *et al.*, 1998), and in light of the mitotic function of HSF2 in bookmarking the hsp70 promoter, we hypothesized that the interaction between

endogenous HSF2 and PRC1 may occur during this stage of the cell cycle. To test this hypothesis, immunoprecipitation analysis was performed using mitotic-enriched populations of HeLa cells. Cells were enriched for mitotic populations by treatment with nocodazole, which depolymerizes microtubules or with taxol, which stabilizes microtubules, and then extracts of these cells were subjected to immunoprecipitation using HSF2 antibodies followed by anti-PRC1 western blot (Figure 3.9A and 3.9B). As an additional approach, cells were transfected with a construct expressing GFP-HSF2, enriched for mitotic cells by nocodazole or taxol treatment, subjected to immunoprecipitation using anti-PRC1 antibodies, followed by western blot using anti-GFP antibodies to detect the transfected GFP-HSF2 protein (Figure 3.10A and 3.10B). The results of this experiment suggest that complexes between PRC1 and both endogenous as well as transfected tagged HSF2 are indeed present in extracts of mitotic cells. In addition, HSF2-PRC1 interaction appears to be independent of microtubule dynamics, as they are observed in extracts of cells treated with nocodazole as well as those of taxol treated cells.

HSF2 and PRC1 co-localize during mitosis

To determine when and where the interaction could be occurring between HSF2 and PRC1 in mitosis with respect to subcellular localization, immunofluorescence analysis was performed. Asynchronous cells were transfected with pEGFP-PRC1 followed by immunofluorescence analysis for endogenous HSF2, with DAPI (4',6 diamidino-2-phenylindole) staining used to identify cells in different stages of mitosis. Previous studies showed that PRC1 localizes predominantly to the mitotic spindle but is inhibited from binding microtubules prior to metaphase in a phosphorylation-dependent manner (Mollinari *et al.*, 2002; Zhu and Jiang, 2005). α -tubulin was co-stained with PRC1 and HSF2 separately to identify the location of the mitotic spindle in relation to these two proteins. GFP-PRC1 and α -tubulin localize (Figure 3.11) as described previously (Mollinari *et al.*, 2002). The results of this experiment indicated that HSF2 and PRC1 appear to co-localize at the periphery of the mitotic spindle at the prometaphase (Prometa) and metaphase (Meta) stages of mitosis but not during anaphase

(Ana) or telophase (Telo) (Figure 3.12). HSF2 and α -tubulin do not appear to co-localize during mitosis (Figure 3.13) which is consistent to the earlier immunoprecipitation data which indicates that the HSF2/PRC1 interaction occurs independently of microtubule dynamics during mitosis.

PRC1 associates with the hsp70 promoter during mitosis

HSF2 has previously been shown to bind to the hsp70 promoter during mitosis to mediate bookmarking of the hsp70 gene promoter (Xing *et al.*, 2005). Since our results showed that HSF2 and PRC1 interact during mitosis, we hypothesized that PRC1 could also be present at the hsp70 promoter. To test this hypothesis, we treated Jurkat cells with nocodazole (Figure 3.14 performed in conjunction with Chad Wilkerson) or taxol (Figure 3.15 performed in conjunction with Chad Wilkerson). Chromatin Immunoprecipitation assay was performed on treated cells using antibodies against PRC1. Precipitated DNA from three independent experiments was analyzed by quantitative PCR using primers specific for the human Hsp70 promoter region. We found that in Jurkat cells treated with nocodazole, PRC1 bound to the Hsp70 promoter approximately 2.2-fold better than the control IgG (Figure 3.14A). Furthermore, in Jurkat cells treated with taxol, PRC1 bound to the Hsp70 promoter approximately 6.2-fold better than the control IgG (Figure 3.15, A). DNA samples from the ChIP assay were also amplified by PCR and analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. The results of this analysis also indicate association of PRC1 with the hsp70 promoter region, and confirm that the PCR products were of the expected size (Figure 3.14B and 3.15B).

Ability of PRC1 to be phosphorylated on threonine 470 and 481 has no effect on the HSF2/PRC1 interaction

An immunoprecipitation experiment was performed to determine if the PRC1 phosphorylation sites play any role in regulating the interaction with HSF2. PRC1 is phosphorylated on threonines 470 and 481 and this phosphorylation is critical to the regulation of the Kif4/PRC1 early in mitosis (Jiang *et al.*, 1998; Mollinari *et al.*, 2002;

Kurasawa *et al.*, 2004; Zhu and Jiang, 2005). Additionally, mutation of these residues has been shown to facilitate premature bundling of microtubules at the mitotic spindle (Mollinari *et al.*, 2002). Based on the previously obtained immunoprecipitation and immunofluorescence data that determined PRC1 and HSF2 interact early in mitosis, prior to when PRC1 is present at the spindle midzone, a logical hypothesis is that PRC1 phosphorylation would regulate the HSF2 interaction since PRC1 is phosphorylated early in mitosis. For this experiment, GFP-tagged PRC1 constructs in which threonines 470 and 481 had been mutated to alanine (GFP-PRC1^{AA}) were generated. Primers for these mutants were previously described and the constructs were characterized and determined to be phosphorylation null mutants (Jiang *et al.*, 1998). For this experiment, HeLa cells were transfected with GFP-PRC1 wild-type or GFP-PRC1^{AA} and nocodazole treated to enrich for mitotic cells. Immunoprecipitation analysis, as described on page 24, was performed using HSF2 antibodies followed by western blot for GFP to detect the PRC1 wild-type or PRC1^{AA} protein. Based on the results in (Figure 3.16), the ability of PRC1 to be phosphorylated has no effect on the interaction with HSF2 which could be explained by the fact that PRC1 and HSF2 interact regardless of microtubule dynamics and PRC1 phosphorylation is known to regulate localization and microtubule bundling ability of PRC1.

HSF2 interaction with PP2A plays a regulatory role in the HSF2/PRC1 interaction

HSF2 interacts with PP2A during mitosis and this interaction is important for hsp70 gene bookmarking. Since PRC1 and HSF2 are both present at the hsp70 promoter, an experiment was designed to determine if the PP2A interaction with HSF2 had any regulatory role for the PRC1 and HSF2 interaction. A previous study determined that amino acids 343-363 in HSF2 were critical for the PP2A interaction and deletion of these residues abolished the HSF2 PP2A interaction (Xing *et al.*, 2007). For this immunoprecipitation analysis, HeLa cells were transfected with GFP-HSF2 wild type or GFP-HSF2 Δ (343-363) which is unable to associate with PP2A. Based on the results of this experiment, loss of PP2A association with HSF2 negatively regulates the interaction with PRC1 (Figure 3.17A.) Figure 3.17B represents the loading controls for PRC1 and β -actin from each transfected cell lysate.

Discussion and Future Directions

With DNA condensation representing a critical step at the beginning of mitosis which mediates the correct segregation of chromosomes, formation of the mitotic spindle is equally important for the process of cytokinesis. Loss of PRC1 has been shown to disrupt cytokinesis and cause an increase in binucleated cells (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). With the characterization of the hsp70 gene bookmarking phenomenon involving HSF2, the question remained as to whether other functions and interactions involving HSF2 in mitosis have yet to be discovered. Consistent with such potential additional mitotic functions of HSF2, a previous study demonstrated that HSF2-/- mouse embryonic fibroblasts (MEFs) exhibited increased numbers of binucleate cells (Paslaru *et al.*, 2003), suggesting that HSF2 function could play a role in the mechanism or regulation of cytokinesis.

This study reports the interaction between HSF2 and PRC1. The results indicate that HSF2 and PRC1 interact during mitosis and that this interaction is specific for HSF2, which is consistent with the hypothesis which suggests that, in addition to bookmarking, HSF2 could have other functions during mitosis. Data obtained from immunoprecipitations using cell lysates treated with nocodazole, a microtubule depolymerizing drug, or taxol, a microtubule stabilizing drug indicate that HSF2 and PRC1 interact independently of microtubule dynamics during mitosis. Previous data indicated that PRC1 does not bind to microtubules during metaphase due to cell cycle-dependent phosphorylation (Jiang *et al.*, 1998; Mollinari *et al.*, 2002). Consistent with the hypothesis that the HSF2/PRC1 interaction is not dependent on microtubule dynamics, the immunofluorescence analysis of untreated cells indicates that HSF2 does not co-localize with α -tubulin during mitosis and that HSF2 and PRC1 localize together at prometaphase and metaphase but not in the later stages of mitosis when PRC1 is localized at the spindle midzone (Jiang *et al.*, 1998; Mollinari *et al.*, 2002; Kurasawa *et al.*, 2004). As mentioned previously, HSF2 is present at the hsp70 promoter during mitosis to mediate bookmarking of the hsp70 gene (Xing *et al.*, 2005). Interestingly, the results of Chromatin Immunoprecipitation analysis in this current study indicate that

PRC1 is also present at the hsp70 promoter during mitosis, suggesting a possible new function for PRC1 in the regulation/mechanism of hsp70 gene bookmarking.

Supporting the hypothesis of a functional complex involving HSF2 and PRC1 is the finding that CAP-G, the subunit of the condensin complex that HSF2 binds to during bookmarking of the hsp70 promoter (Xing *et al.*, 2005), also interacts with Kif4, a kinesin-4 family member that has been shown to interact with and translocate PRC1 along the mitotic spindle to bundle interdigitating microtubules (Kurasawa *et al.*, 2004; Mazumdar *et al.*, 2004; Zhu and Jiang, 2005). The interaction between Kif4 and PRC1 is regulated by phosphorylation of PRC1 (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005). Early in mitosis both PRC1 and Kif4 are present at the mitotic spindle, when PRC1 is phosphorylated, but these two proteins do not interact at that time (Zhu and Jiang, 2005). Dephosphorylation of PRC1 allows it to interact with Kif4, and as a result Kif4 is then able to translocate PRC1 onto microtubules where PRC1 bundles the microtubules to facilitate formation of the midzone, a necessary component for cytokinesis (Zhu and Jiang, 2005). Except for the phosphorylation of PRC1, little is known regarding interacting partners or additional regulation of PRC1 prior to metaphase as the interaction between Kif4 does not occur until the metaphase to anaphase transition.

Interestingly, a previous study showed that Kif4 interacts with PRC1 at the amino terminus of the protein (Kurasawa *et al.*, 2004), the same region of PRC1 that our studies indicate is involved in interactions with HSF2 (reference Figure 3.1). Thus, one possibility in elucidating the biological role that this interaction plays is that HSF2 could be responsible for negatively regulating PRC1 early in mitosis by binding to the same region in PRC1 with which Kif4 interacts, thereby mediating the lack of PRC1-Kif4 interaction at that stage. Since PRC1 is phosphorylated early in mitosis, PP2A which also interacts with HSF2 could potentially dephosphorylate PRC1 and regulate the interaction with Kif4. The phosphatase responsible for PRC1 dephosphorylation is not known although it is hypothesized that Cdc14A or PP1 γ is responsible (Zhu and Jiang, 2005). Alternatively, the interaction between Kif4 and CAP-G could function as an inhibitory mechanism to prevent HSF2 from interacting with CAP-G and prevent dephosphorylation of CAP-G by HSF2 associated PP2A which could cause unnecessary dysregulation of the condensin enzyme. Future studies to determine if HSF2 interacts

with Kif4 early in mitosis could provide striking data that expands the mechanism of hsp70 bookmarking or elucidates a previously undefined role for HSF2 in the regulation of mitosis.

Recent studies have also reported that PRC1 acts as a docking site for Plk1 in a phosphorylation-dependent manner (Neef *et al.*, 2007; Santamaria *et al.*, 2007). Previous analysis of PRC1 speculated that a subpopulation of PRC1 is present at the spindle midzone but does not bundle microtubules leading to the hypothesis that PRC1 could be part of protein complex at the spindle midzone (Mollinari *et al.*, 2002). Although HSF2 and PRC1 interact in the early stages of mitosis, a similar hypothesis that PRC1 and HSF2 are part of a protein complex at the beginning of mitosis leads to an intriguing question for future studies to determine if HSF2 acts in a similar manner, as PRC1 does for Plk1, to bring PRC1 into proximity with an as yet undetermined binding partner at the hsp70 promoter.

Figure 3.1A Schematic of the HSF2-interacting region of PRC1.

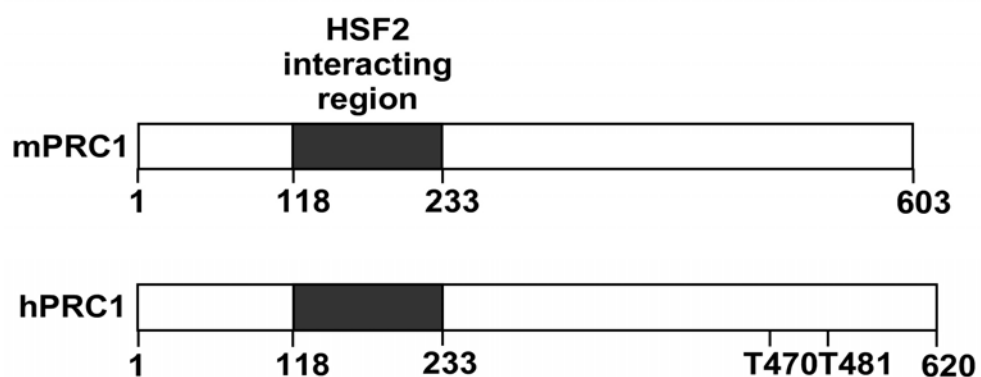


Figure 3.1A HSF2-interacting region of PRC1. Schematic showing the location of the HSF2-interacting region of PRC1 identified by the yeast two-hybrid analysis, which comprises amino acids 118-233 of mouse PRC1 which is homologous to amino acids 118-233 of human PRC1.

Nucleotide

Protein

Figure 3.1B Nucleotide and Protein alignment of *Mus musculus* PRC1(mPRC1) to *Homo sapiens* PRC1(hPRC1) (Accession # BC003138). For the nucleotide sequence, the mPRC1 sequence from the yeast two-hybrid analysis was aligned with hPRC1. The nucleotide sequence of mPRC1 is 82% identical to hPRC1. For the protein alignment, the mPRC1 sequence was translated and aligned against the hPRC1 sequence and is also 82% identical based on protein sequence.

Figure 3.2 Yeast two-hybrid analysis of HSF2 interaction with PRC1.

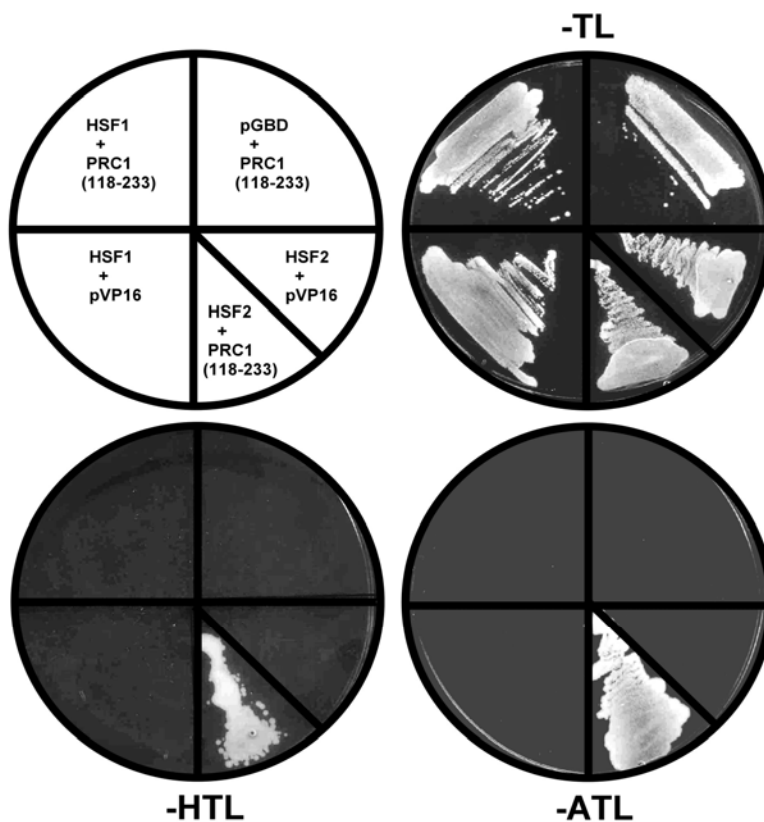


Figure 3.2 Yeast two-hybrid analysis of HSF2 and PRC1. Yeast strain pJ694A transformed with pGBD-HSF2 or pGBD-HSF1 along with either pVP16 PRC1(118-233) or pVP16 alone (or pGBD alone with pVP16 PRC1(118-233)) were streaked on plates lacking tryptophan and leucine (-TL), tryptophan, leucine, and histidine (-HTL), or tryptophan, leucine, and alanine (-ATL).

Figure 3.3 β -galactosidase assay between HSF2, HSF1, and PRC1.

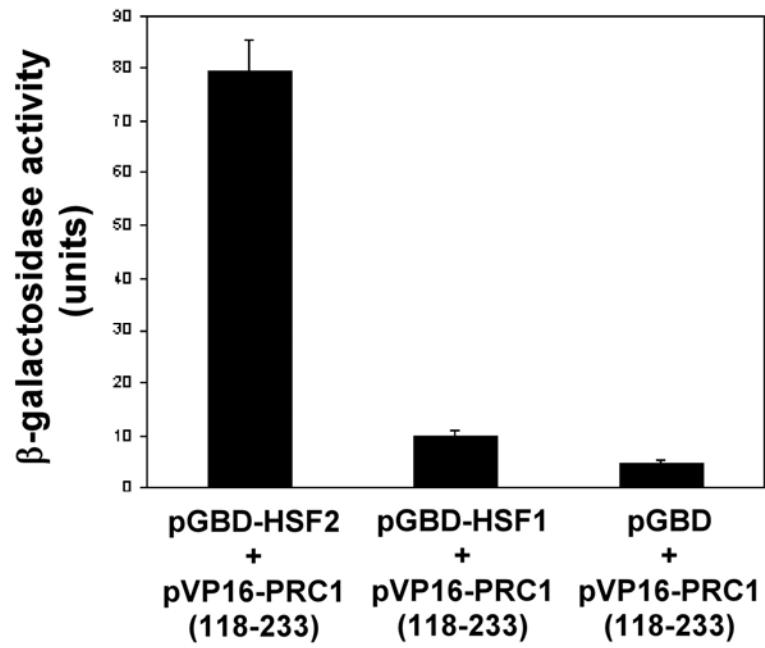


Figure 3.3 β -galactosidase activity between HSF2/HSF1 and PRC1.

β -galactosidase activity was measured in extracts of yeast strain pJ694A transfected with pVP16 PRC1(118-233) along with either pGBD-HSF2, pGBD-HSF1, or empty pGBD-C1 vector.

Figure 3.4 Yeast two-hybrid screen for the PRC1 interacting region of HSF2.

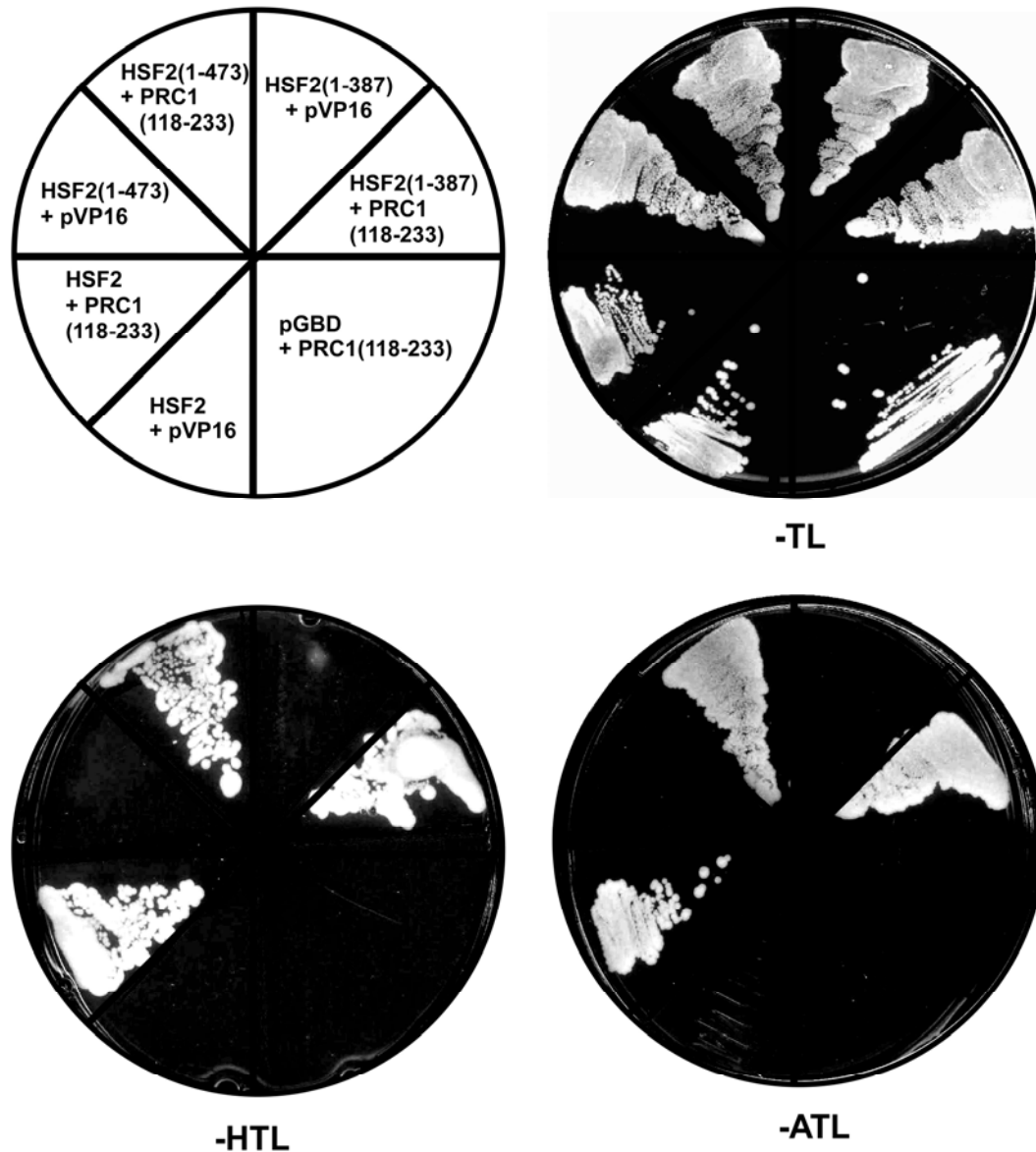


Figure 3.4 Yeast two-hybrid screen of HSF2 deletion mutants and PRC1. Yeast strain pJ694A transformed with pGBD-HSF2 full length, pGBD-HSF2 (1-473), pGBD-HSF2 (1-387), along with either pVP16 PRC1(118-233) or pVP16 alone (or pGBD alone with pVP16 PRC1(118-233)) were streaked on plates lacking tryptophan and leucine (-TL), tryptophan, leucine, and histidine (-HTL), or tryptophan, leucine, and alanine (-ATL).

Figure 3.5 β -galactosidase assay of HSF2 deletion mutants and PRC1.

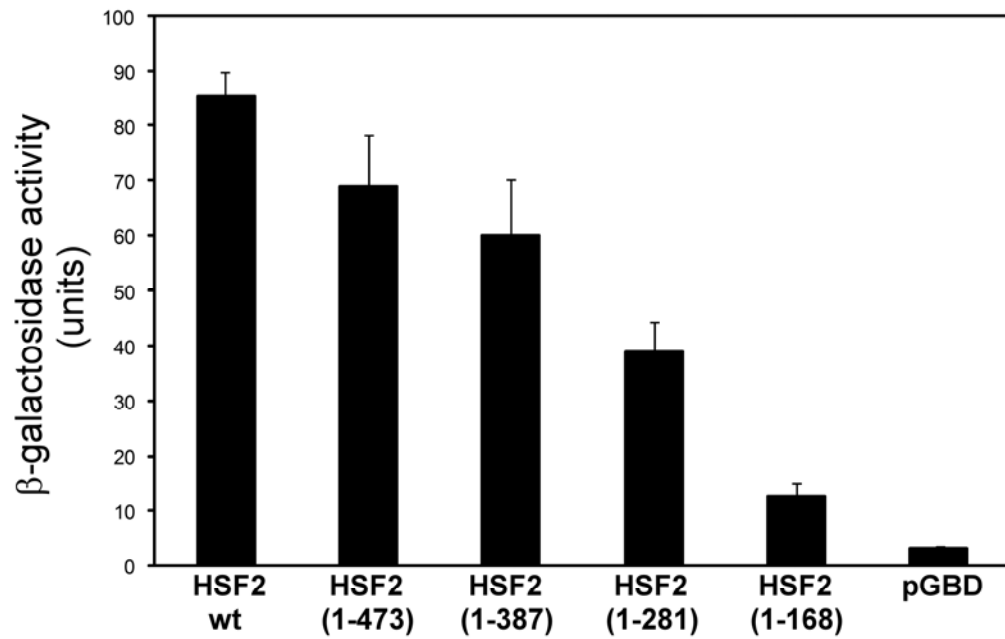


Figure 3.5 β -galactosidase assay between HSF2 deletion mutants and PRC1. β -galactosidase activity was measured in extracts of yeast strain pJ694A transfected with pVP16 PRC1(118-233) along with either pGBD-HSF2, pGBD-HSF2 (1-473), pGBD-HSF2 (1-387), pGBD-HSF2 (1-281), pGBD-HSF2 (1-168), or empty pGBD-C1 vector.

Figure 3.6 Schematic of putative PRC1 interacting region of HSF2.

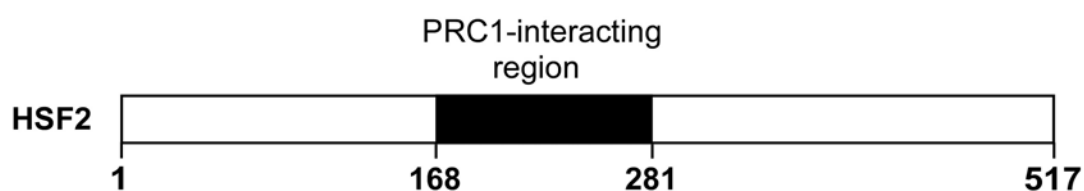


Figure 3.6 PRC1-interacting region of HSF2. Schematic showing the location of the predicted PRC1-interacting region of HSF2, which comprises amino acids 168-281 of HSF2, identified by the β -galactosidase assay of HSF2 deletion mutants..

Figure 3.7 In vitro binding analysis between PRC1 and HSF2.

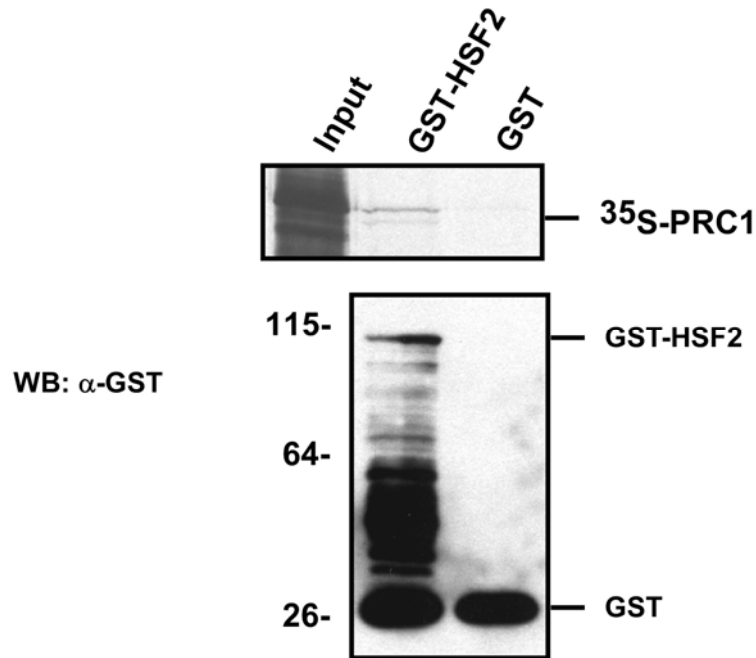


Figure 3.7 Interaction between HSF2 and *in-vitro* translated PRC1. ^{35}S -labeled *in vitro* translated full length human PRC1 was incubated with GST-HSF2 or GST that were bound to glutathione-agarose beads. After washing, the amount of bound ^{35}S -labeled full length human PRC1 was determined by SDS-PAGE and autoradiography. Amounts of GST-HSF2 and GST bound to beads were determined by GST Western blot. Input represents 8% of total translated ^{35}S -label PRC1.

Figure 3.8A. Characterization of Bethyl PRC1 antibody.

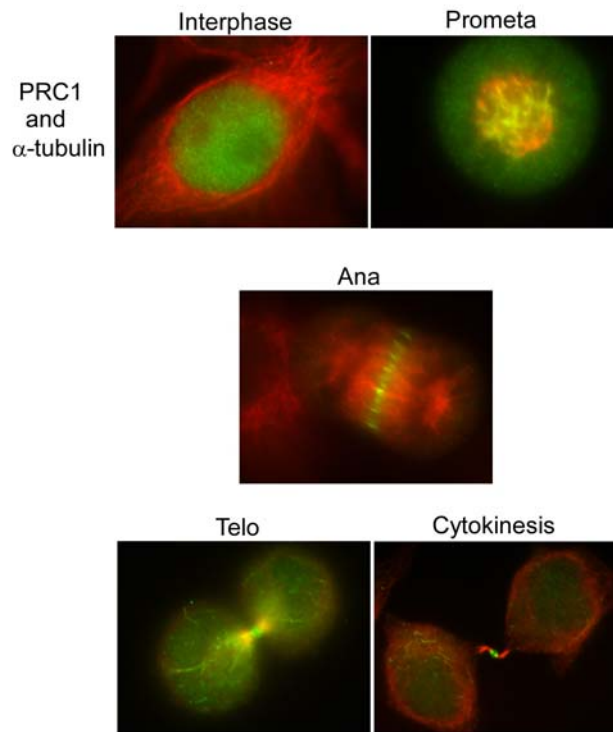


Figure 3.8A Characterization of the Bethyl PRC1 antibody. Cells were subjected to immunofluorescence analysis using anti α -tubulin antibodies (Sigma-Aldrich) or anti-PRC1 antibodies (Bethyl) followed by incubation with Texas Red anti-mouse IgG (Vector Laboratories) or Alexa Fluor 488 anti-goat antibodies. Cells were visualized using a 100x oil immersion objective on a Nikon fluorescent microscope. Endogenous PRC1 (green) was co-stained with α -tubulin (red) as a positive control for the location of the mitotic spindle throughout mitosis.

Figure 3.8B. PRC1 interaction is specific for HSF2 in mitotic cell lysates.

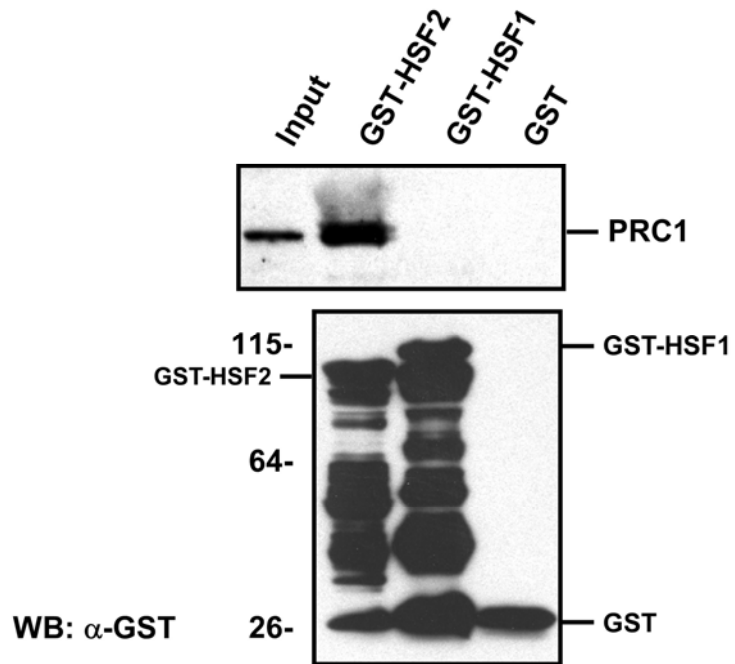


Figure 3.8B *In vitro* binding analysis between endogenous PRC1 and HSF2. Lysates of nocodazole-blocked HeLa cells were incubated with GST-HSF2, GST-HSF1 or GST bound to glutathione agarose. After washing, the amount of bound PRC1 was determined by SDS-PAGE followed by Western blot using goat polyclonal anti-PRC1 antibodies (Bethyl). Input represents 2% of total cell lysate used.

Figure 3.9 HSF2 Immunoprecipitation for interaction with PRC1 during mitosis.

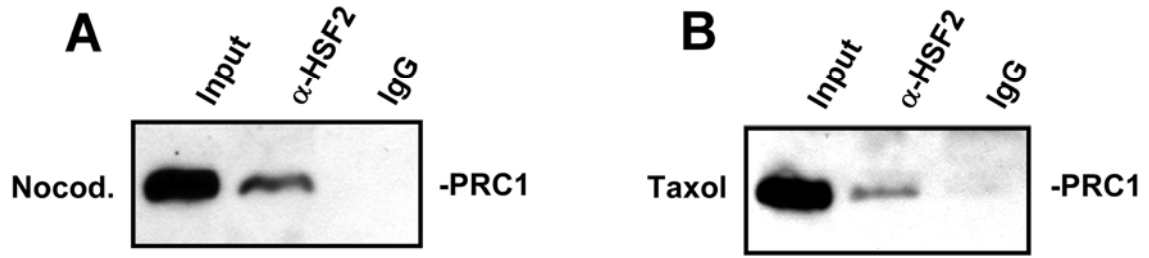


Figure 3.9 HSF2 interacts with PRC1 during mitosis. Extracts of nocodazole (nocod.) (250 ng/ml for 18 hours) (A) or taxol treated (10 nM for 24 hours) (B) HeLa cells were subjected to immunoprecipitation analysis using anti-HSF2 or non-specific IgG, followed by Western blot analysis of the immunoprecipitates using anti-PRC1 antibodies. Inputs represent 2% of total cell lysate used.

Figure 3.10 PRC1 Immunoprecipitation for interaction with GFP-HSF2 during mitosis.

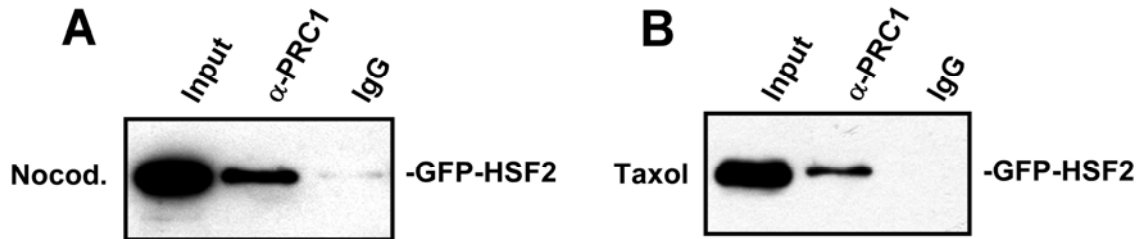


Figure 3.10 HSF2 interacts with PRC1 during mitosis. HeLa cells were transfected with pEGFP-HSF2 using Effectene (Qiagen) or JetPEI (Bridge Bioscience) according to manufacturer's instructions. Extracts of nocodazole (nocod.) (250 ng/ml for 18 hours) (A) or taxol treated cells were prepared (10 nM for 24 hours) (B). Cells were then subjected to immunoprecipitation analysis using anti- PRC1 antibodies or non-specific IgG, followed by Western blot analysis of the immunoprecipitates using anti-GFP antibodies. Inputs represent 2% of total cell lysate used.

Figure 3.11 PRC1 and α -tubulin localization during mitosis.

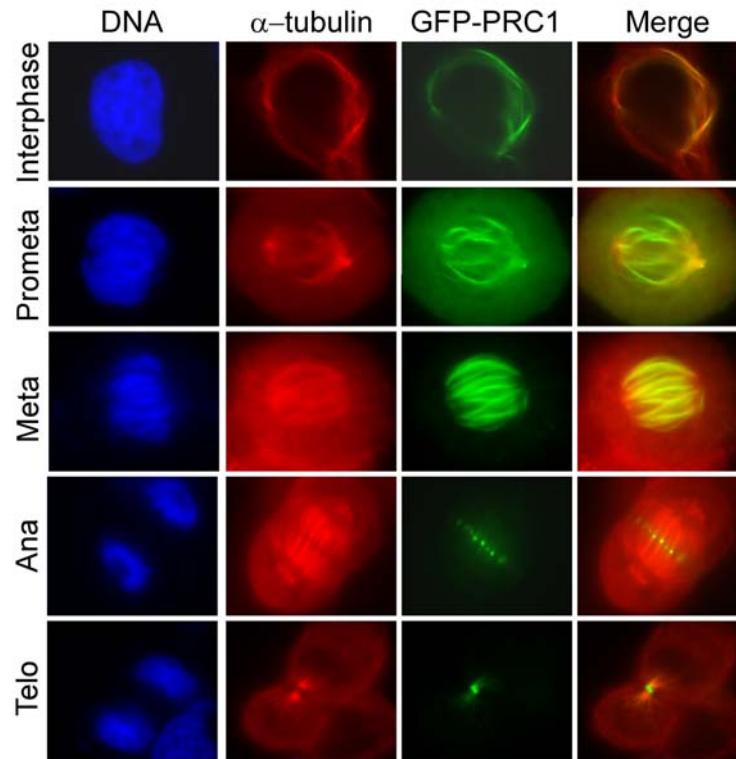


Figure 3.11 Localization between GFP-PRC1 and α -tubulin during mitosis. HeLa cells were transfected with pEGFP-PRC1. After transfection, cells were subjected to immunofluorescence analysis using anti α -tubulin antibodies (Sigma-Aldrich) followed by incubation with Texas Red anti-mouse IgG (Vector Laboratories). Cells were visualized using a 100x oil immersion objective on a Nikon fluorescent microscope. GFP-PRC1 was co-stained with α -tubulin as a positive control for the location of the mitotic spindle throughout mitosis.

Figure 3.12 HSF2 and α -tubulin do not appear to localize together in mitosis.

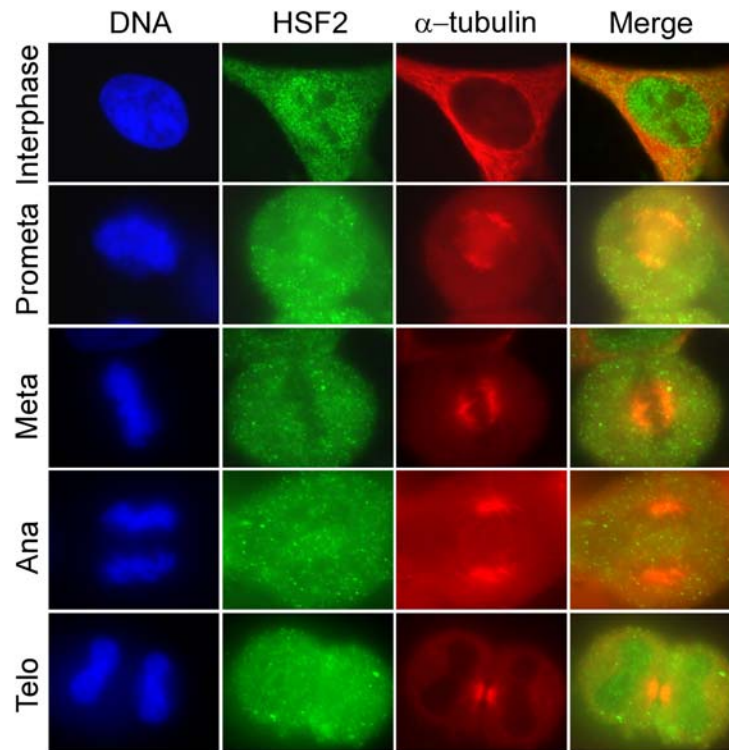


Figure 3.12 HSF2 and α -tubulin do not localize together in mitosis. Untransfected HeLa cells were subjected to immunofluorescence analysis using either anti-HSF2 (Bethyl) or anti α -tubulin antibodies (Sigma-Aldrich) followed by incubation with Texas Red anti-mouse IgG (Vector Laboratories) or Alexa Fluor® 488 donkey anti-goat IgG. Cells were visualized using a 100x oil immersion objective on a Nikon fluorescent microscope.

Figure 3.13 HSF2 and PRC1 localize together during prometaphase and metaphase of mitosis.

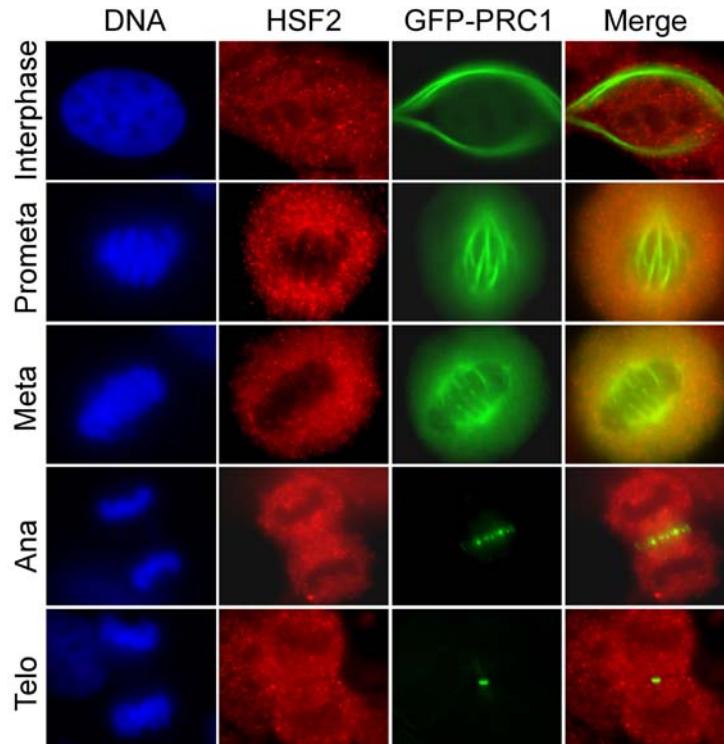


Figure 3.13 HSF2 and PRC1 co-localize during mitosis. HeLa cells were transfected with pEGFP-PRC1. After transfection, cells were subjected to immunofluorescence analysis using either anti-HSF2 (Bethyl) followed by incubation Alexa Fluor® 594 donkey anti-goat IgG (Invitrogen). Cells were visualized using a 100x oil immersion objective on a Nikon fluorescent microscope. GFP-PRC1 and HSF2 appear to co-localize during prometaphase and metaphase but not in the later stages of mitosis.

Figure 3.14 PRC1 is associated with the hsp70 promoter during mitosis.

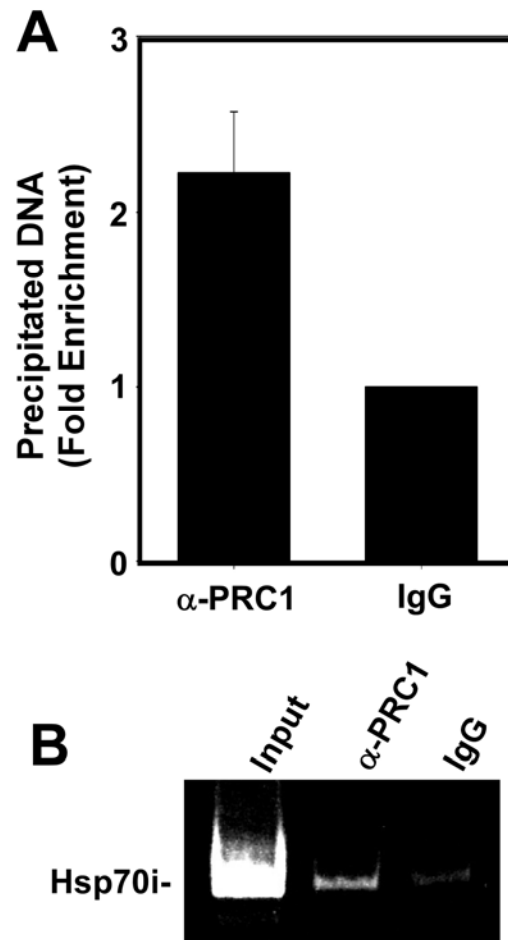


Figure 3.14 PRC1 is present at the hsp70 promoter in nocodazole treated cells. Jurkat cells treated with nocodazole were subjected to ChIP analysis using antibodies against PRC1 or IgG (negative control) (*Experiments performed in conjunction with Chad Wilkerson*). Precipitated DNA, amplified using primers specific to the Hsp70 promoter, was quantitated by real time PCR (Figure 2.14A). The data represent the results of three independent ChIP assays, and error bars represent the standard error of the means. Precipitated DNA was also amplified with the same Hsp70 primers on a Robocycler PCR instrument. Amplified products were resolved by polyacrylamide gel electrophoresis and bands detected by ethidium bromide staining (Figure 2.14B).

Figure 3.15 PRC1 is present at the hsp70 promoter in taxol treated cells.

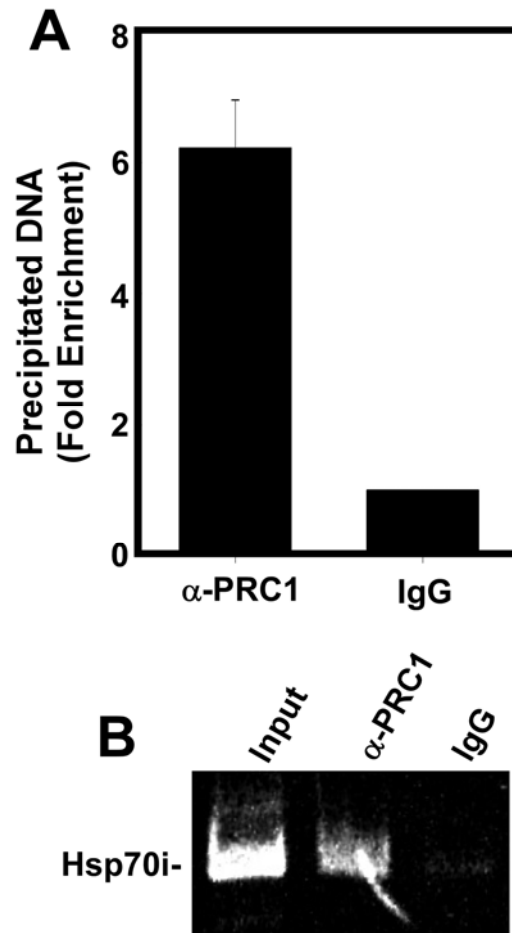


Figure 3.15 PRC1 is associated with the Hsp70 promoter during mitosis. Jurkat cells treated with taxol were subjected to ChIP analysis using antibodies against PRC1 or IgG (negative control) (*Experiments performed in conjunction with Chad Wilkerson*). Precipitated DNA, amplified using primers specific to the Hsp70 promoter, was quantitated by real time PCR (Figure 2.15A). The data represent the results of three independent ChIP assays, and error bars represent the standard error of the means. Precipitated DNA was also amplified with the same Hsp70i primers on a Robocycler PCR instrument. Amplified products were resolved by polyacrylamide gel electrophoresis and bands detected by ethidium bromide staining (Figure 2.15B).

Figure 3.16. Ability of PRC1 to be phosphorylated does not effect the interaction with HSF2.

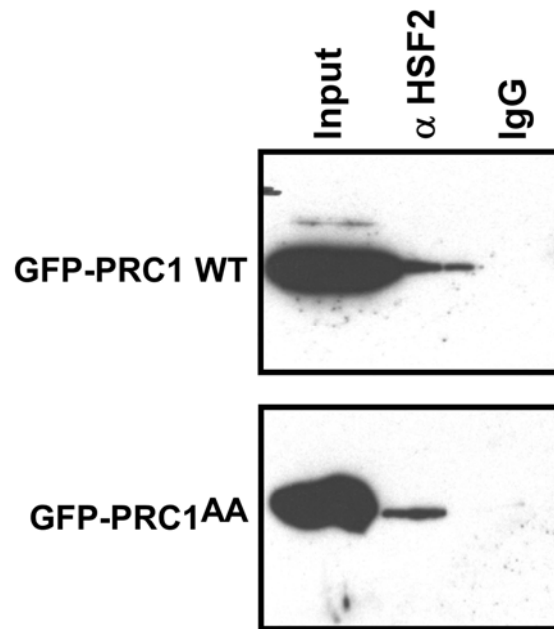


Figure 3.16. PRC1 phosphorylation has no effect on the interaction with HSF2.

HeLa cells were transfected with pEGFP-PRC1 or pEGFP-PRC1^{AA} using Effectene (Qiagen) according to manufacturer's instructions. Cells were enriched for mitotic cells using nocodazole (250 ng/ml for 18 hours). Cells were then subjected to immunoprecipitation analysis using anti- HSF2 antibodies or non-specific IgG, followed by Western blot analysis of the immunoprecipitates using anti-GFP antibodies. Input represents 2% of total cell lysate used.

Figure 3.17A and 3.17B. HSF2 interaction with PP2A appears to regulate PRC1 interaction.

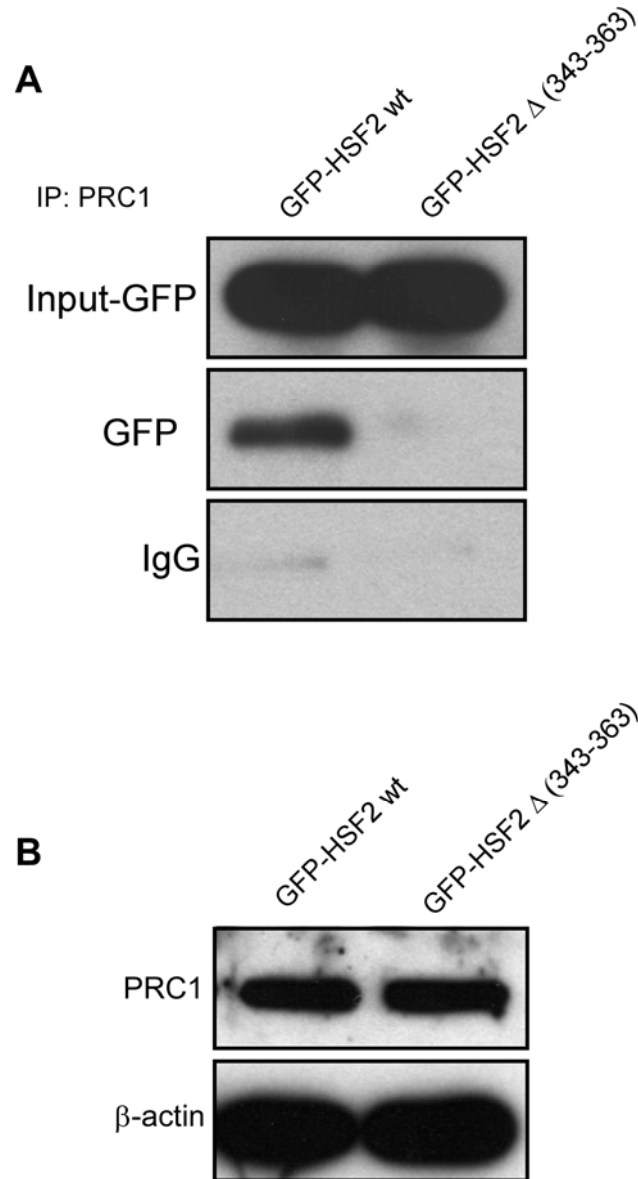


Figure 3.17A and 3.17B. PP2A interaction with HSF2 regulates PRC1 interaction. HeLa cells were transfected with pEGFP-HSF2 or pEGFP-HSF2 Δ (343-363) using Effectene (Qiagen) according to manufacturer's instructions. Lysates were prepared from cells that were nocodazole treated (250 ng/ml for 18 hours). Cells were then subjected to immunoprecipitation analysis using anti- PRC1 antibodies or non-specific IgG, followed by Western blot analysis of the immunoprecipitates using anti-GFP antibodies (Figure 3.17A). Figure 3.17B represents PRC1 and β -actin western blots which were used as loading controls for the immunoprecipitation.

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VITA

Lynea Alene Murphy
Birthplace: Wise, VA
June 21, 1979

EDUCATION

1997-2001: University of Virginia's College at Wise, Wise, VA.
B.S. Biology (summa cum laude)
1997: John I. Burton High School, Norton, VA.

PROFESSIONAL POSITIONS

2002-2008: Graduate Research Assistant, University of Kentucky
1998-2001: Laboratory Assistant/Teaching Assistant, Department of Natural Sciences,
University of Virginia's College at Wise

SCHOLASTIC AND PROFESSIONAL HONORS

2008-present: Ohio Valley Society of Toxicology (OVSOT)
2007-present: Translating Research to Youth through Information Technology Program
(TRY-IT), Youth Mentor
2007-2008: University of Kentucky, Graduate Center for Toxicology, Admissions
Committee Member
2002-2006: Training Grant Recipient of Ruth L. Kirschstein National Research
Service Award
2001: Smiddy Award for Outstanding Senior in Natural Sciences, University of
Virginia's College at Wise
1997-2001: C. Bascom Slemple Foundation Grant
1997-2001: University of Virginia's College at Wise, Full Academic Scholarship

PROFESSIONAL PUBLICATIONS

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- L.A. Murphy** and K.D. Sarge. Characterizing the interaction between PRC1 and HSF2 During Mitosis. Eli Lilly Convention Center. Indianapolis, Indiana OVSOT. 2007. (Poster presentation)
- D.C. Wilkerson, **L.A. Murphy**, and K.D. Sarge. Investigating the Role of HSF2 during Spermatogenesis. The 40th Annual Meeting of the Society for the Study of Reproduction. San Antonio, TX. 2007. (Poster presentation)
- D.C. Wilkerson, **L.A. Murphy**, and K.D. Sarge. HSF2 Bookmarks the Hsp70 Gene in Epididymal Spermatozoa. The 26th Annual Symposium in Reproductive Sciences. University of Kentucky. Lexington, KY. 2007. (Poster presentation)
- L.A. Murphy** and K.D. Sarge. Interaction between HSF2 and PRC1: Implications for hsp70 regulation and cytokinesis. University of Kentucky. Department of Molecular and Cellular Biochemistry Annual Retreat. Pineville, KY. 2007. (Poster presentation)
- L.A. Murphy**. Alumni seminar: Invited speaker. The University of Virginia's: College at Wise. Wise, VA. 2007. (Oral presentation)
- L.A. Murphy** and K.D. Sarge. HSF2: Interaction and Regulation of Protein Regulating Cytokinesis 1 (PRC1) During Mitosis. University of Kentucky. Department of Molecular and Cellular Biochemistry Annual Retreat. Pineville, KY. 2006. (Oral presentation)
- L.A. Murphy** and K.D. Sarge. Role of HSF2 Interaction with Protein Regulating Cytokinesis 1 (PRC1) During Mitosis. Clinical and Translational Science Spring Conference. University of Kentucky. College of Medicine. Lexington Civic Center. Lexington, KY. 2006. (Poster presentation)
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Lynea Alene Murphy